

(19)



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(11)

EP 0 655 890 B1

(12)

EUROPEAN PATENT SPECIFICATION(45) Date of publication and mention
of the grant of the patent:**26.01.2005 Bulletin 2005/04**(51) Int Cl.7: **C12N 15/55**, C12N 1/19,
C12N 9/16, C12N 15/81(21) Application number: **93918426.3**(86) International application number:
PCT/US1993/007058(22) Date of filing: **27.07.1993**(87) International publication number:
WO 1994/003072 (17.02.1994 Gazette 1994/05)(54) **RECOMBINANT CELLS, DNA CONSTRUCTS, VECTORS AND METHODS FOR EXPRESSION
PHYTATE DEGRADING ENZYMES IN DESIRED RATIOS**REKOMBINANTE ZELLE, DNA-KONSTRUKTIONEN, VEKTOREN UND METHODEN ZUR
EXPRESSION VON PHYTATABBAUENDEN ENZYMEN IN GEWÜNSCHTEN VERHÄLTNISSENCELLULES RECOMBINEES, STRUCTURES D'ADN, VECTEURS ET METHODES POUR
EXPRIMER DES ENZYMES DE DEGRADATION DE PHYTATE SELON DES RAPPORTS REQUIS(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IE IT LI NL PT SE(74) Representative: **Hiebl, Inge, Dr. et al**
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Patent- und Rechtsanwälte
Thomas-Wimmer-Ring 15
80539 München (DE)(30) Priority: **31.07.1992 US 925401**(43) Date of publication of application:
07.06.1995 Bulletin 1995/23(56) References cited:
EP-A- 0 449 375 EP-A- 0 619 369
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Description

[0001] This application is a continuation-in-part of U.S. application Serial No. 07/925,401 filed July 31, 1992.

5 Field of the Invention

[0002] This invention is related to strains of filamentous fungi capable of over-expressing at least two phytate degrading enzymes in desired ratios. Also disclosed are DNA sequences, promoters, DNA constructs and vectors useful for the preparation of such strains.

10 Background of the Invention

[0003] Minerals are essential elements for the growth of all organisms. For livestock production of monogastric animals (e.g., pigs, poultry) and fish, feed is commonly supplemented with minerals. Plant seeds are a rich source of minerals since they contain ions that are complexed with the phosphate groups of phytic acid. Ruminants do not require inorganic phosphate and minerals because microorganisms in the rumen produce enzymes that catalyze conversion of phytate (myo-inositol-hexaphosphate) to inositol and inorganic phosphate. In the process minerals that have been complexed with phytate are released.

[0004] Phytate occurs as a source of stored phosphorus in virtually all plant feeds (for a review see: *Phytic Acid, Chemistry and Applications*, E. Graf (Ed.), Pilatus Press: Minneapolis, MN, U.S.A., 1986). Phytic acid forms a normal part of the seed in cereals and legumes. It functions to bind dietary minerals that are essential to the new plant as it emerges from the seed. When the phosphate groups of phytic acid are removed by the seed enzyme phytase, the ability to bind metal ions is lost and the minerals become available to the plant. In livestock feed grains, the trace minerals bound by phytic acid are only partially available for absorption by monogastric animals, which lack phytase activity. Although some hydrolysis of phytate occurs in the colon, most phytate passes through the gastrointestinal tract of monogastric animals and is excreted in the manure contributing to fecal phosphate pollution problems in areas of intense livestock production. Inorganic phosphorus released in the colon has no nutritional value to livestock because inorganic phosphorus is absorbed only in the small intestine. Thus, a significant amount of the nutritionally important dietary minerals are potentially not available to monogastric animals.

[0005] Conversion of phytate to inositol and inorganic phosphorus can be catalyzed by microbial enzymes referred to broadly as phytases. Phytases such as the phytase #EC 3.1.3.8 are capable of catalyzing hydrolysis of myo-Inositol hexaphosphate to D-myo-inositol 1,2,4,5,6-pentaphosphate and orthophosphate. Certain fungal phytases reportedly hydrolyze inositol pentaphosphate to tetra-, tri-, and lower phosphates; e.g., *A. ficuum* phytases reportedly produce mixtures of myoinositol di- and mono-phosphate (Ullah, 1988). Phytase producing microorganisms comprise bacteria such as *Bacillus subtilis* (V.K. Powar and V.J. Jagannathan, *J. Bacteriol.* **151**:1102-1108, 1982) and *Pseudomonas* (D. J. Cosgrove, *Austral. J. Biol. Sci.* **23**:1207-1220, 1970); yeasts such as *Saccharomyces cerevisiae* (N.R. Nayini and P. Markakis, *Lebensmittel Wissenschaft und Technologie* **17**:24-26, 1984); and fungi such as *Aspergillus terreus* (K. Yamada, Y. Minoda and S. Yamamoto, *Agric. Biol. Chem.* **32**:1275-1282, 1968). The possible use of microbes capable of producing phytase as a feed additive for monogastric animals has been reported previously (Shieh and Ware, U.S. Patent No. 3,297,548; Nelson, T.S. et al., *J. Nutrition* **101**:1289-1294, 1971). To date, however, commercial application of this concept has not proved feasible, because of the high cost for production of microbial phytases.

[0006] Microbial phytases may also reportedly be useful for producing animal feed from certain industrial processes, e.g., wheat and corn waste products. The wet milling process of corn produces glutens sold as animal feeds. Addition of phytase may reportedly improve the nutritional value of the feed product. Fungal phytase enzymes and process conditions (t ~ 50°C and pH ~ 5.5) have been reported previously in European Patent Application 0 321 004. In processing soybean meal the presence of phytate reportedly renders the meal and wastes unsuitable for feeds used in rearing fish, poultry and other non-ruminants as well as calves fed on milk. Phytase is reportedly useful for improving the nutrient and commercial value of this high protein soy material (see *Finase Enzymes* By Alko, a product information brochure published by Alko Ltd., Rajamaki, Finland). A combination of phytase and a pH2.5 optimum acid phosphatase from *A. niger* has been used by Alko, Ltd as an animal feed supplement in their phytic acid degradative product *Finase F* and *Finase S*. A cost-effective source of phytase would greatly enhance the value of soybean meal as an animal feed (Shieh et al., 1969).

[0007] Phytase and less specific acid phosphatases are produced by the fungus *Aspergillus ficuum* as extracellular enzymes (Shieh et al., 1969). Ullah reportedly purified a phytase from wild-type *A. ficuum* that had an apparent molecular weight of 61.7 kDa (on SDS-PAGE; as corrected for glycosylation); pH optima at pH2.5 and pH5.5; a Km of about 40 µM; and, a specific activity of about 50U/mg (Ullah, A., *Preparative Biochem.* **18**:443-458, 1988); PCT patent application WO 91/05053 also reportedly discloses isolation and molecular cloning of a phytase from *Aspergillus ficuum* with pH optima at pH2.5 and pH5.5, a Km of about 250 µM, and specific activity of about 100U/mg protein.

[0008] Acid phosphatases are enzymes that catalytically hydrolyze a wide variety of phosphate esters and usually exhibit pH optima below 6.0 (Hollander, 1971); e.g., #EC 3.1.3.2 catalyzes hydrolysis of orthophosphoric monoesters to orthophosphate products. An acid phosphatase has reportedly been purified from *A. ficuum*. The deglycosylated form of the acid phosphatase has an apparent molecular weight of 32.6 kDa (Ullah et al., 1987).

[0009] Objects of the invention provide recombinant phosphatases isolated from filamentous fungi that improve the efficiency of release of phosphorus from phytate and the salts of phytic acid. Other objects of the invention provide efficient and inexpensive sources of two or more recombinant enzymes that are suitable for commercial use in feeds and industrial processes with minimal processing.

Summary of the Invention

[0010] Two enzymes were substantially purified from *Aspergillus niger* var. *awamori* strain ALKO243: namely, a phytase and a pH2.5 acid phosphatase. The amino acid sequence for isolated internal peptides was determined for each enzyme and the deduced nucleotide sequences were used to construct probes that were used to molecularly clone the two genes. The genomic nucleotide sequence was determined for each gene and the coding region sequence was also determined (where necessary) by cloning the cDNA. Comparison of the deduced amino acid sequences of the phytase and pH2.5 acid phosphatase with other known phosphatases identified a potential enzyme active site sequence. Transformation vectors were constructed using promoters native to filamentous fungi and several different heterologous promoter sequences were compared in their ability to drive expression of the respective genes. Recombinant host cells were selected that over-produced phytase and pH2.5 acid phosphatase at levels that were about 2-fold to about 4000-fold higher, and 10-fold to 126-fold higher (respectively), than the levels of these enzymes secreted by the ALKO243 (ATCC#38854) strain of *Aspergillus*. Dual-gene-transformed host cells were also selected that expressed elevated levels of the two recombinant enzymes, i.e., both phytase and pH2.5 acid phosphatase. Selected strains of dual-gene-transformed cells were identified that synthesized and secreted phytase along with pH2.5 acid phosphatase within desired tailor-made ranges of the respective enzyme activities, e.g., within a range of 3:1 to 16:1 pH2.5 acid phosphatase activity to phytase activity. The transformed recombinant host cells disclosed herein solve the problems in the prior art and provide cost-effective sources of commercial phosphatase enzymes suitable for use in commercial processes that liberate minerals from phytates in plant materials either *in vitro*, i.e., in feed treatment processes, or *in vivo*, i.e., by administering one or more of the enzymes to animals.

[0011] The phytase purified from *A. niger* var. *awamori* strain ALKO243 (ATCC#38854; also known as IFO4033) exhibited an apparent molecular weight of 80-86,000 daltons (SDS-PAGE), and 45,000-48,000 daltons (SDS-PAGE), following treatment with endoglycosidase F/N-glycosidase F. The purified phytase appears to be monomeric under nondenaturing conditions with an isoelectric point of approximately 5.3. This phytase enzyme exhibits activity in the absence of metal ions, i.e., metal ion independent; the enzyme has a pH optima of about 5.0; and, a temperature optimum in the range of 55-60°C.

[0012] The purified pH2.5 acid phosphatase from *A. niger* var. *awamori* strain ALKO243 had an apparent molecular weight of about 66,000 daltons (SDS-PAGE) and 46,000-49,000 daltons (SDS-PAGE) after removal of carbohydrates with endoglycosidase F/N-glycosidase F. This purified pH2.5 acid phosphatase appears to be tetrameric under non-denaturing conditions with an isoelectric point of approximately 4 to 4.25; an apparent K_m of 0.7mM for sodium phytate at pH 2.5 and 4mM for paranitrophenylphosphate; a pH optima of about pH2.5; and, temperature optimum of about 55°C.

[0013] Translated nucleotide sequences for phytase and pH2.5 acid phosphatase yielded polypeptides of 470 amino acids and 479 amino acids, respectively. The calculated molecular weights for the predicted phytase and pH2.3 acid phosphatase polypeptides were approximately 51,400 daltons and approximately 52,700 daltons, respectively.

[0014] The desired ratio of recombinant pH2.5 acid phosphatase phytase and phytase produced by a dual-gene-transformed recombinant host cells achieves a balanced enzyme mixture in which cooperative enzyme activity rapidly and effectively catalyzes the near complete hydrolysis of phytate to inositol and free phosphate with release of minerals from the phytic acid complex.

[0015] The present invention therefore relates to a recombinant transformed host cell characterized in that it has been transformed by a first nucleic acid capable of hybridising under stringent conditions with the nucleotide sequence of SEQ ID NO. 18 and a second nucleic acid capable of hybridising under stringent conditions with the nucleotide sequence of SEQ ID NO. 20 and that it is capable of secreting the pH 2.5 acid phosphatase enzyme activity and the phytase enzyme activity in a ratio of 3:1 to 16:1.

Brief Description of the Drawings

[0016] The foregoing aspects and many of the attendant advantages of this invention will become more readily appreciated as the same becomes better understood by reference to the following detailed description, when taken in

conjunction with the accompanying drawings, wherein:

FIGURE 1 shows the pH optima of a metal ion independent phytase enzyme purified from *Aspergillus niger* var. *awamori* strain ALKO243 (ATCC#38854) with a pH optima of about pH5; and, >20% maximal enzyme activity in the range of pH2 to pH7 (as described in Example 1, below);

FIGURE 2 shows the temperature optima at 55-60°C, with >20% of maximal enzyme activity in the range of 25°C to 65°C, for the purified phytase enzyme of FIGURE 1, above;

FIGURE 3 shows the pH optima at 37°C of pH2.5 acid phosphatase purified from *Aspergillus niger* var. *awamori* strain ALKO243 that has an apparent Km of 0.7mM for sodium phytate at pH2.5, a pH optima of pH2.0-2.5, and >20% activity in the range of pH1.5 to pH3.5 (as described below in Example 1);

FIGURE 4 shows the temperature optima at about 55°C, and >20% maximal enzyme activity in the range of 25°C to 60°C, for the pH2.5 acid phosphatase of FIGURE 3, above;

FIGURE 5 shows the relative phytase activity (i.e., liberation of phosphate from sodium phytate) of Finase, a commercial preparation containing phytase and phosphatases, as a function of pH at 37°C (squares) and 55°C (+);

FIGURE 6 shows the relative phytase activity (i.e., phosphate-liberating activity) of Finase (FIGURE 5) as a function of temperature, i.e., between 10°C and 70°C, at pH5;

FIGURE 7 shows an autoradiogram of radiolabeled degenerate oligonucleotide phytase probe PHY-1 hybridizing under stringent conditions with endonuclease fragments of genomic DNA generated with BamHI; EcoRI; XbaI; BamHI + EcoRI; BamHI + XbaI; and EcoRI - XbaI (as described in Example 2, below);

FIGURE 8 shows an autoradiogram of radiolabeled specific oligonucleotide pH2.5 acid phosphatase probe PHY-31 hybridizing under stringent conditions with endonuclease fragments of genomic DNA generated with BamHI, HindIII, KpnI, PstI, SalI, SphI, or SstI (as described in Example 2, below);

FIGURE 9A and FIGURE 9B (SEQ. ID. NO. 18) shows the genomic nucleotide sequence for the phytase gene in *Aspergillus niger* var. *awamori* strain ALKO243 (ATCC#38854); the deduced amino acid sequence (SEQ. ID. NO. 19) for the phytase polypeptide, as described in Example 2, below; and, the 5' regulatory promoter region of the gene;

FIGURE 10A, FIGURE 10B, FIGURE 10C and FIGURE 10D show the organization of phytase expression vector constructs pFF1, pFF2, pFF3 and pFF4 (as described in Example 2, below);

FIGURE 11A and FIGURE 11B (SEQ. ID. NO. 20) shows the nucleotide sequence for the pH2.5 acid phosphatase gene and the deduced amino acid sequence (SEQ. ID. NO. 21) of the enzyme;

FIGURE 12A, FIGURE 12B, FIGURE 12C and FIGURE 12D shows the organization of phytase expression vector constructs pFF-6A, pFF8, pFF9, and pFF1 that were designed to remove any *E. coli* nucleotide sequences (as described in Example 4, below);

FIGURE 13A, FIGURE 13B, FIGURE 13C and FIGURE 13D shows the organization of pH2.5 acid phosphatase vector constructs pPHO-1-4A from which linear fragments can be isolated as described in Example 4, below;

FIGURE 14A and FIGURE 14B shows the organization of two transformation vectors having both the pH2.5 acid phosphatase gene and phytase gene, namely, pFIN-1A and pFIN-1B (as described in Example 4, below);

FIGURE 15 shows phytase activity in a plate assay of transformed *A. niger* var. *awamori* subclones that over-produce phytase by up to 1260-fold over the levels produced by the parental ALKO243 strain. (The size of the circles formed by the molybdate indicator complex around clones is proportional to enzyme activity; as described in Example 4, below);

FIGURE 16 shows chromosomal phytase gene copy number and mRNA levels in phytase gene transformed *A. niger* var. *awamori* strain ALKO2268 as determined by Southern blot analysis and Northern analysis, respectively (as described in Example 4, below). The significant increase in phytase activity seen in FIGURE 15 in transformed cells is attributable to integration of one or more copies of the cloned recombinant phytase gene construct into chromosomal DNA (see Example 4, below);

FIGURE 16A shows Southern blot analysis of untransformed control ALKO2268 (lanes 1, 4 and 7) and over-producing phytase transformants (lanes 2, 5, 8, 9 and 10);

FIGURE 16B shows Northern blot analysis of mRNA levels in untransformed Control ALKO2268 (lane 1) and transformed strains (lanes 2-6);

FIGURE 17 and FIGURE 18 show chromosomal phytase and pH2.5 acid phosphatase gene copy numbers and mRNA levels in dual-gene-transformed *A. niger* var. *awamori* strain ALKO243 as determined by Southern blot analysis and by Northern analysis, respectively (as described in Example 5);

FIGURE 17A shows Southern blot analysis of untransformed control ALKO243 (lane 2) and dual-transformed strains (lanes 3, 4 and 5) probed for phytase genes;

FIGURE 17B shows Northern blot analysis of untransformed control (lane 1) and dual-transformed strains (lanes 2, 3 and 4) probed for phytase mRNA;

FIGURE 18A shows Southern blot analysis of untransformed control ALKO243 (lane 3) and dual-transformed

strains (lanes 4, 5 and 6) probed for pH2.5 acid phosphatase genes;
 FIGURE 18B shows Northern blot analysis of untransformed control ALKO243 (lane 1) and dual-transformed strains (lanes 2, 3 and 4) probed for pH2.5 acid phosphatase mRNA;
 FIGURE 19 graphically depicts the levels of phytase activity in twenty-four different transformants using circular or linear plasmid vector constructs (as described in Example 6, below); and
 FIGURE 20 shows the effects of different promoters on the levels of recombinant phytase activity and pH2.5 acid phosphatase activity in dual-gene-transformed *A. niger* var. *awamori* strains ALKO243 and ALKO2268, as described in Example 6, below.

Detailed Description of the Preferred Embodiment

[0017] As used herein the following terms are intended to mean as follows:

[0018] The term "phosphatase" is intended to mean an enzyme capable of releasing phosphate from a phosphate-containing substrate, e.g., phytate. Representative examples of phosphatases include fungal phytases and acid- and neutral-phosphatases such as pH2.5 acid phosphatase and pH6 neutral phosphatase.

[0019] The term "nucleic acid" is used herein to refer to natural or synthetic DNA and RNA, polynucleotides (i.e., greater than three nucleotides), and oligonucleotides (i.e., greater than nine nucleotides).

[0020] The term "capable of hybridizing under stringent conditions" is used herein to mean annealing to a subject nucleotide sequence, or its complementary strand, under standard conditions, e.g., high temperature and/or low salt content which tends to disfavor annealing of unrelated sequences. A suitable protocol (involving 0.1X SSC and annealing at 68°C for 2 hours) is described in Maniatis, T. et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, Cold Springs Harbor, N.Y., pp. 387-389, 1982.

[0021] The term "nucleotide sequence" is used herein to refer to a sequence of nucleotides or nucleosides and may include non-contiguous sequences; e.g., in genomic sequences exon coding region sequences may be interrupted by intron sequences and the like.

[0022] The term "contiguous nucleotide sequence" is used herein to refer to a sequence of nucleotides linked in a serial array, one following the other.

[0023] The term "coding region" refers to the nucleotide sequence within a nucleic acid that when transcribed and translated will give rise to a subject amino acid sequence, e.g., exon regions in genomic DNA that when transcribed into mRNA will direct translation of the subject amino acid sequence. The term "encoded" is used to mean an amino acid sequence coded for in the triplet code of nucleotides by the coding region nucleotide sequence.

[0024] The term "transformation vector" is used herein interchangeably with "vector construct" to refer to a recombinant construct (e.g., a plasmid DNA) containing a subject phytase, and/or pH2.5 acid phosphatase nucleotide sequence incorporated therein, and includes as one subclass "expression vectors". Representative examples of transformation vectors include plasmid vectors of *E. coli* (e.g., *pBR322*, *pUC18*, *pUC19* and the like as well as vectors useful for transforming filamentous strains of fungi (e.g., *pLO-3*, *pFF-6*, *pPHO-1* and the like). The subject phytase or pH2.5 acid phosphatase nucleotide sequence may contain the regulatory sequence elements from the 5' region of the respective gene (i.e., termed herein "native regulatory elements"); or, a heterologous promoter (i.e., from a strain, variety, species or genus other than *A. niger* var. *awamori* strain ALKO243 or from a different gene, e.g., GA or GAPDH promoter nucleotide sequences) may be added to drive expression of the subject nucleotide sequence. The subject transformation vector may also contain suitable restriction sites for receiving additional nucleotide sequences useful for promoting chromosomal integration of the vector DNA. The subject transformation vectors are capable of introducing phosphatase nucleotide sequences into a host cell so that they may integrate effectively into the host cell DNA, and so that the product of the coding region of the nucleotide sequence is expressed by the transformed host cell.

[0025] "Promoter" is used to mean a nucleotide sequence capable of promoting expression of a downstream nucleotide sequence such as transcription and translational regulatory signal sequences and the like. Representative examples of promoters are provided below in Examples 3-5, e.g., GA, GAPDH, and native phytase or pH2.5 acid phosphatase promoter.

[0026] "Selectable marker" is used to mean a nucleotide sequence capable of encoding a polypeptide that when expressed by transformed cell confers upon the cell the ability to be identified or selected from among the other cells in a population of cells. Illustrative examples include antigenic markers, phenotypic markers (e.g., cell size, shape, budding patterns and the like), and drug resistance markers such as phleomycin resistance.

[0027] As used herein the term "transformed recombinant host cell" refers to a cell having chromosomal DNA with an integrated transformation vector nucleotide sequence therein. Representative examples of transformed recombinant host cells of the invention include bacterial and fungal cells capable of synthesizing and/or secreting a recombinant phytase and/or one or more recombinant phosphatases, i.e., a phytase encoded by a nucleotide sequence capable of hybridizing under stringent conditions with SEQ. ID NO 18; and, the recombinant pH2.5 acid phosphatase, i.e., a phosphatase encoded by a nucleotide sequence capable of hybridizing with SEQ. ID. NO. 20.

[0028] The term "phytase nucleotide sequence" as used herein refers to the nucleotide sequence located within SEQ. ID. NO. 18.

[0029] The term "pH2.5 acid phosphatase nucleotide sequence" as used herein refers to a nucleotide sequence capable of hybridizing under stringent conditions with a nucleotide sequence in SEQ. ID. NO. 20.

[0030] The term "phosphatase" as used herein encompasses all enzymes capable of cleaving a phosphate ester bond in a substrate, and includes phytases, and acid and neutral phosphatases.

[0031] The term "phytase" is intended to mean an enzyme capable of hydrolyzing a phosphate ester bond in a phytate substrate, e.g., inositolhexaphosphate, inositolpentaphosphate, inositoltetraphosphate, and inositoltriphosphate and salts thereof

[0032] The term "purified phytase" is intended to mean a purified enzyme of the invention isolated and substantially purified from *A. niger* var. *awamori* strain ALKO243. The subject enzymes are monomeric under nondenaturing conditions with isoelectric points of about 5.3; apparent molecular weights on SDS-PAGE of about 80,000 daltons to 86,000 daltons; apparent molecular weights after removal of carbohydrate (e.g., with endoglycosidase F/N-glycosidase F) of about 45,000-48,000 daltons (SDS-PAGE). The subject purified phytase enzymes have the following catalytic properties: namely, the enzymes are metal ion-independent enzymes capable of hydrolyzing a phosphate ester bond in a sodium phytate substrate. The subject enzymes have a pH optima of about pH5 at 37°C; exhibit greater than 20% of maximal enzyme activity in the range of pH2 to pH7; and, have a temperature optima at 55°C to 60°C with >20% maximal enzyme activity in the range of 25°C to 65°C. One unit of phytase activity (PU) is the amount of enzyme protein required to liberate 1nmol of inorganic phosphate from sodium phytate in one minute at 37°C under the assay conditions described in Example 1, below.

[0033] The term "recombinant phytase" is intended to mean a phytase enzyme encoded by the nucleotide coding sequence of FIGURES 9A and 9B; SEQ. ID. NO. 18 and produced by a recombinant host cell transformed with the subject transformation vector containing the subject phytase nucleotide sequence. The molecular weight predicted from transcription of the coding region of subject phytase nucleotide sequence is a polypeptide translation product (i. e., prior to glycosylation) of about 51,000 daltons to about 52,000 daltons.

[0034] The term "metal ion independent" is intended to mean that the activity of the enzyme can be measured in the absence of Mn^{2+} , Mg^{2+} and Ca^{2+} . However, this is not intended to mean that the activity of the enzyme may not be increased in the presence Mn^{2+} , Mg^{2+} or Ca^{2+} .

[0035] The term "acid phosphatase" is used to mean an enzyme having a pH optimum for mediating hydrolysis of a phosphate ester bond in a substrate at a pH less than pH5.0, preferably less than pH3.0.

[0036] The term "pH2.5 acid phosphatase" is used to refer to a phosphatase having a pH optimum for mediating hydrolysis of a phosphate ester in a substrate at pH2.0-2.5, e.g., a sodium phytate substrate.

[0037] The term "purified pH2.5 acid phosphatase" is intended to mean a purified enzyme of the invention isolated and substantially purified from *A. niger* var. *awamori* strain ALKO243. When purified from the subject strain of filamentous fungi the enzyme has the following properties: namely, the enzyme isolated under nondenaturing conditions is a glycoprotein tetrameric complex of four identical monomers with an apparent isoelectric point of about 4.0 to about 4.25. Each of the monomers has an apparent molecular weight of about 66,000 daltons under denaturing conditions in SDS-PAGE, and the monomers have an apparent molecular weight of 46,000-49,000 daltons (SDS-PAGE) after substantial removal of carbohydrate. The subject purified pH2.5 acid phosphatase enzymes have the following catalytic properties: namely, the enzymes can catalyze hydrolysis of a phosphate ester bond in a sodium phytate substrate with an apparent K_m of about 0.7mM (i.e., at 37°C and pH2.5, as described in Example 1, below). The subject enzymes have a pH optima of about pH2 to about pH2.5 (i.e., at 37°C); have greater than 20% of maximal enzyme activity in the range of pH1.5 to pH3.5; and, have a temperature optima at 55°C with >20% maximal enzyme activity in the range of 25°C to 60°C. One unit of pH2.5 acid phosphatase activity (HFU) is the amount of enzyme protein required to liberate 1nmol of inorganic phosphate from p-nitrophenyl phosphate in one minute at 37°C under the conditions described in Example 1, below.

[0038] The term "recombinant pH2.5 acid phosphatase" is intended to mean a pH2.5 acid phosphatase enzyme encoded by the coding region of a nucleotide sequence capable of hybridizing under stringent conditions with a nucleotide sequence in FIGURES 11A and 11B; SEQ. ID. NO. 20 and produced by a recombinant host cell transformed with the subject transformation vector containing the subject pH2.5 acid phosphatase nucleotide sequence. In a representative example, the subject pH2.5 acid phosphatase nucleotide sequence is the coding region of SEQ ID. NO. 20 that encodes a polypeptide with an apparent molecular weight (i.e., prior to glycosylation) of about 52,000 daltons to about 53,000 daltons.

[0039] The term "secreted" is used herein to refer to the extracellular form of a protein, e.g., a "secreted" protein that is synthesized in a cell and transported into the extracellular culture medium where its presence or enzyme activity may be assayed.

[0040] The term "phytase enzyme activity" is used herein to refer to the catalytic activity of a phytase, e.g., in mediating hydrolysis of a sodium phytate substrate to release phosphate, as may be conveniently measured in an assay for

phosphate such as that described in the Examples, below.

[0041] The term "pH2.5 acid phosphatase enzyme activity" is used herein to refer to the phosphatase activity of a pH2.5 acid phosphatase, e.g., in mediating hydrolysis of a phosphate ester bond in a paranitrophenylphosphate substrate (in an assay such as that described in the Examples, below).

[0042] The term "over-producing" is used interchangeably with the term "over-expressing" to indicate that the subject transformed recombinant host cell is capable of synthesizing and secreting levels of the subject enzyme that are at least about 2-fold higher than the amount of enzyme synthesized under identical conditions by cells of *A. niger* var. *awamori* strain ALKO243 (ATCC#38854). As illustrated in Example 4, (Tables 8 and 9, below), overproduction in strain ALKO2268 results in secretion in shake flask cultures conducted according to Example 1 of about 45-fold more phytase enzyme activity per ml of the culture media than that secreted under equivalent conditions by ALKO243. Illustrative transformants with the subject nucleic acids of the invention (i.e., also shown in Example 4) produce about 6-fold greater activity than ALKO2268 (i.e., see Table 7; up to about 300-fold greater activity than ALKO243). Other transformants in Example 4 (i.e., Tables 8 and 9) produced phytase activities per ml up to about 2,100-fold greater than ALKO243. Over-production in respect to pH2.5 acid phosphatase is similarly illustrated by transformants in Example 4, below, that produced up to about 130-fold (i.e., Table 11) greater activity per ml than ALKO243. By comparison, strain ALKO2268 produces approximately 41-46% of the pH2.5 acid phosphatase enzyme activity produced by ALKO243.

[0043] The term "ratio of pH2.5 acid phosphatase to phytase" refers to the ratio of a pH2.5 acid phosphatase enzyme activity to a phytase activity, in this case, a ratio that may be calculated by dividing the enzyme activity of pH2.5 acid phosphatase per ml of sample (e.g., the number of HFU/ml) by the phytase enzyme activity per ml of sample (e.g., the number of PU/ml). Representative assays for determining phytase and pH2.5 acid phosphatase activity are provided in the Examples, below. For comparative purposes, the results presented in the Examples below show the amount of phytase and pH2.5 acid phosphatase activity, respectively, produced and secreted into the culture broth by *A. niger* var. *awamori* strain ALKO243 (ATCC#38854) over 5 days of fermentation culture under the conditions described in Tables 7 and 8, below (Example 3). Cells of ALKO243 cultured under these conditions produce about 80-450PU of phytase and about 5,000-6,000HFU of pH2.5 acid phosphatase; and, for reference purposes, the over-producer phytase strain *A. niger* var. *awamori* strain ALKO2268 produces about 3,000-9,000PU and 2,000-3000HFU. With these exemplary strains the ratio of pH2.5 acid phosphatase to phytase (i.e., HFU/PU) is about 0.6 in shake flask fermentation culture media obtained from ALKO2268 after 5 days, and is 64.7 with ALKO243, (e.g., see Tables 11 and 14-16, below). As illustrated in Example 5, below, ratios of pH2.5 acid phosphatase activity (HFU) to phytase activity (PU) ranged from about 4 to about 16 with transformants shown in Table 14, and about 3 to 6 with the transformants whose enzymes activities are shown in Tables 15 (PU) and Table 16 (HFU). The subject "ratio of pH2.5 acid phosphatase to phytase" achieves a balanced enzyme mixture in which cooperative enzyme activity rapidly and effectively catalyzes the near complete hydrolysis of phytate to inositol and free phosphate with release of minerals from the phytic acid complex at a pH (e.g., that in the stomach of a monogastric animal) and temperature desired in a commercial product. The term "cooperative enzyme activity" is used to mean that the subject ratios confer upon the mixture of enzymes the properties of: a) more rapid catalysis of phytate to inositol and free phosphate; b) more efficient conversion of phytate to inositol and free phosphate; and, c) more complete conversion of phytate (i.e., IP6, see the Examples below) to inositol and phosphate (i.e., greater than 80% conversion as illustrated in Example 1, Table 1, below).

[0044] One "phytase normalized unit" or "PNU" is defined as the amount of phytase activity produced by the *A. niger* ALKO243, which in this case is equivalent to 85 PU/ml. One "acid phosphatase normalized unit" or "APNU" is defined as the amount of acid phosphatase activity produced by the *A. niger* ALKO243 strain, which in this case is equivalent to 5500 HFU/ml.

[0045] Embodiments of the invention provide phytase nucleotide sequences and also pH2.5 acid phosphatase nucleotide sequences capable of hybridizing under stringent conditions with a nucleotide sequence of SEQ. ID. NO 18 or SEQ. ID. NO. 20, respectively. The subject nucleotide sequences are useful for constructing oligonucleotide probes, transformation vectors, transformed recombinant host cells, encoding recombinant phytase and pH2.5 acid phosphatase proteins, and the like, as described below.

[0046] In other embodiments the invention provides transformed recombinant host cells, e.g., *E. coli*, *Bacillus*, *Aspergillus*, *Trichoderma*, *Penicillium*, *Cephalosporium*, *Rhizopus*, and the like, that have copies of the subject phytase and/or pH2.5 acid phosphatase nucleotide sequences of the invention. In a preferred embodiment the transformed recombinant host cells are selected from among species of filamentous fungi, e.g., *Aspergillus*, *Trichoderma*, and *Rhizopus*; and, in another preferred embodiment the subject host cells are selected from among varieties and strains of *Aspergillus niger*. In a most preferred embodiment the subject host cells are selected from among recombinant host cells having phytase nucleotide sequences, e.g., transformed cells of the phytase over-producing strains GAI-6, GAL-142, GAN-1, GAG-12, GAO-248, GAI-12, GAK4-46, GAI-2, GAK4-52, GAM-111, GAK4-47, GAM-225, GAD-103, GAD-23, GAD-103, GAD-23, GAD-130, CAM-199, GAE-3, GAE-32, GAM-111, and GAL-65 (as described in Example 4, below). In another preferred embodiment the subject host cells are selected from among recombinant host cells trans-

formed with vectors having pH2.5 acid phosphatase nucleotide sequences, e.g., transformed cells of strains GAO-69, GAW-131, GBL-128, GBL-97, GAO-61, GAW-89, GAW-130, GAW-121, GBL-87, GBL-119, GAO-84, GAW-54, GBL-129, GAW-141, GBL-103, GAW-112, GBL-92, GAW-114, and GAT-143 (as described in Example 2, 4, 5, or 6 below). Other preferred host cells include cells selected from the transformed strains GAX-11, GAX-12, GBE-14, GBH-134, GBH-15, GBJ-9, GBJ-10, GBJ-13, GBJ-16, GBJ-26, GBJ-27, GBJ-28, GBJ-31, GBJ-35, GBJ-38, GBJ-40, GBJ-76, and GBJ-82 (as described below in Example 2, 4, 5, or 6). Those skilled in the art will recognize that the nucleotide sequences, transformation vectors and transformed recombinant host cells provided herein are useful for identifying additional strains having substantially the same properties as the afore-identified strains.

[0047] Skilled artisans will recognize that the nucleotide sequences and deduced amino acid sequences of the invention may be useful in constructing nucleotide and antibody probes for identifying and isolating natural variants, and mutants of transformants; e.g., mutants resulting from treatment with chemicals, UV, gamma-irradiation and the like). Mutants may have increased expression of the subject phytase, pH2.5 acid phosphatase, or both a phytase and a pH2.5 acid phosphatase. Representative screening assay for identifying the latter mutants include Northern and Southern blotting, and Western blotting with antibodies directed to peptides (natural and synthetic) within the deduced amino acid sequences of the subject phytases and pH2.5 acid phosphatases.

[0048] Those skilled in the art will of course recognize that mixtures of transformed recombinant host cells may be used to achieve production of a phytase and one or more phosphatases; e.g., transformed recombinant host cells from a strain producing phytase may be mixed with transformed recombinant host cells from a strain producing pH2.5 acid phosphatase. In this manner, the mixed cell cultures may be constructed so that the cells release a desired ratio of phytase enzyme activity to pH2.5 acid phosphatase enzyme activity.

[0049] The subject transformed recombinant host cells of the invention may also be used for preparing substantially pure recombinant phytase preparations. In a preferred embodiment phytase nucleotide sequences in the transformed recombinant host cell encode a polypeptide having a phytase amino acid sequence RHGXRX, wherein R is arginine, H is histidine, G is glycine, X is any amino acid, and P is proline.

[0050] Embodiments of the invention also provide "mixtures" of recombinant phytase and pH2.5 acid phosphatase in varying states of purity and formulated in a desired ratio of substantially pure pH2.5 acid phosphatase to phytase enzyme activity. Formulating such a balanced mixture of enzymes in the desired ratios confers upon the mixture the property of cooperative enzyme activity (described above) that can be tailor-made to encompass the range of properties desired in a selected commercial application (e.g., uses such as those described below). Starting material for the recombinant phytase and recombinant pH2.5 acid phosphate include fermentation broths, production culture media, and the like from transformed recombinant host cells or from selected strains that over-produce the subject phytase and/or pH2.5 acid phosphatase. Down-stream processing of the subject enzymes into a product may involve removal of cells and cellular debris (e.g., by centrifugation, filtration and the like), followed by concentration (e.g., by ultrafiltration, ion exchange or affinity chromatography and the like), or the starting material may be suitable for use in commercial processes after a simple purification (e.g., lyophilization). The subject mixtures may be prepared by combining equal (or different) amounts of the subject enzyme preparations (e.g., from different cell cultures), in order to achieve the desired ratio of the respective enzyme activities, or the subject mixtures may be existent in the same culture (e.g., the product of a dual-gene-transformed recombinant host cell, as described below). In a preferred embodiment the subject mixture contains a ratio of a phosphatase enzyme activity (e.g., pH2.5 acid phosphatase) to a phytase enzyme activity that is about 3:1 to about 16:1.

[0051] Embodiments of the invention provide transformed recombinant host cells that are constructed with one or more transformation vectors having a phytase nucleotide sequence and one or more phosphatases, i.e., a pH2.5 acid phosphatase nucleotide sequence, wherein expression of each enzyme under the control of an independent promoter sequence. The subject transformed host cells are selected for expression of the two (or more) protein products within a desired range of enzyme activities.

[0052] The phosphatases produced by the transformed recombinant host cells and processes that are embodiments of the invention provide the following advantages over other enzyme preparations used previously in the art: namely, the subject phosphatases of the invention have higher enzyme activity per unit volume of sample, greater yield of enzyme protein per unit volume of sample, greater cost-efficiency, less concentration and/or purification required for use in a commercial product or process, greater efficacy in converting phytate to free inositol and inorganic phosphate, and fewer digestive side-effects when used in animal feeds.

[0053] The subject phosphatases produced by the transformed recombinant host cells of the invention are useful in commercial processes for releasing minerals from complexes with phytate in plant materials such as seeds and waste matter of milling, e.g., soybean meal, so that low value materials are converted efficiently and effectively to a high quality feed for non-ruminant animals. Examples of commercial processes in which the subject enzymes may be useful include corn wet milling, plant protein isolation (especially soy protein isolation), cereal treatment for use in baking, and the like.

[0054] In a preferred embodiment the subject phosphatases of the invention are added directly to animal feeds so

that phosphates are ingested by the animal and released in vivo in the digestive tract, e.g., of a non-ruminant animal. In this case the subject phosphatases are selected to have pH optima for their respective enzyme activities that coincide with the digestive pH encountered in a non-ruminant animal (i.e., the range of pH1 to pH6).

[0055] Suitable methods for preparing the subject enzymes for use in products include spray drying, stabilization in liquid formulations, granulation, and encapsulation, which are known to those skilled in the art.

[0056] The subject phosphatase enzymes of the invention, and subject mixtures of enzymes, are capable of degrading phytate to free phosphate more efficiently and rapidly than any one of the constituent enzymes alone. Embodiments of the invention provide a mixture of a phytase and a pH2.5 acid phosphatase that is capable of degrading inositol-phosphates of phytates and phytic acids, inositolhexaphosphate, IP6; inositolpentaphosphate, EP5; inositoltetraphosphate, IP4; inositoltriphosphate, IP3; inositol diphosphate, IP2; inositol monophosphate, IP1; to free inositol and inorganic phosphate. The subject mixture provides cooperative enzyme activities by constructing an enzymatic cascade for more rapid, complete, and efficient conversion of a phytate substrate to inorganic phosphate and inositol. For example, a phytase having phytate (IP6) as a preferred substrate may catalyze efficient hydrolysis of IP6 to IP5, IP4, IP3, and IP2 but not to free inositol and inorganic phosphate. In turn, a pH2.5 acid phosphatase may prefer simple phosphate substrates (e.g., IP5, IP4, IP3, IP2 and IP1) and may catalyze efficient hydrolysis of these substrates to free inositol and inorganic phosphates. It is believed that by formulating the subject phosphatase of the invention within desired optimum ranges of phytase to pH2.5 acid phosphatase activity the subject mixtures of the invention provide balanced enzyme mixtures having cooperative enzyme activity. The subject mixtures of the invention formulated in this manner may provide more rapid, efficient and complete release of greater amounts of free inositol and inorganic phosphate from phytate and phytic acid than produced in the same time (and under the same conditions of pH and temperature) by either of the constituent phosphatase enzymes.

EXAMPLE 1

Purification and amino acid sequencing of phosphatases: phytase and acid-phosphatase peptides

[0057] [General materials and methods are described in the section entitled "*Materials and Methods*", which follows at the end of this and each subsequent Example.]

Purification of phytase and pH2.5 acid phosphatase:

[0058] Phytase and pH2.5 acid phosphatase enzymes were purified at 4°-8°C (unless otherwise stated) from the cell-free culture medium nitrate/concentrate of *A. niger* var. *awamori* strain ALKO243 (ATCC#38854). The culture nitrate/concentrate (990ml) was adjusted (on ice) to 70% saturation in ammonium sulfate, and the precipitate was removed by centrifugation at 10,000xg for 15 min. The supernatant (1070ml) was next separated by hydrophobic chromatography on Octyl-Sepharose CL-4B (Pharmacia). The column (5cm × 17cm) was equilibrated in a 20mM bis-Tris/HCl buffer, pH6.2, containing 0.436g (NH₄)₂SO₄ per ml; supernatant was applied; and, non-adsorbed proteins were removed by washing with 500ml of the equilibration buffer solution. Adsorbed proteins were eluted from the column using a 500ml linear gradient from 70% to 0.0% of ammonium sulfate in 20mM bis-Tris/HCl buffer, pH6.2. Ten ml fractions were collected and analyzed for phytase and pH2.5 acid phosphatase enzyme activity. Most phytase activity eluted early in the gradient. Fractions containing the respective different enzyme activities were pooled separately; concentrated by ultrafiltration on Amicon PM10 membrane filters. Phytase containing fractions were desalted by passage over PD10 (Pharmacia) gel filtration columns equilibrated in 50mM bis-Tris/HCl, pH6.2 buffer. Phytase purification is described first, followed by acid phosphatase purification.

[0059] Phytase was purified first by anion exchange chromatography on DEAE-Sepharose (Pharmacia). Briefly, a 24.5ml aliquot was applied to a 5cm x 7cm column equilibrated in 50mM bis-Tris/HCl, pH6.2. Non-adsorbed proteins were removed by washing with the equilibration buffer (100ml), and adsorbed proteins were eluted using a linear 200ml gradient from 0.0M to 0.5M NaCl, in equilibration buffer. The fractions were assayed for phytase activity and fractions with activity were pooled and concentrated to 600μl using a Centricon-30 miniconcentrator. Portions of 100μl were applied at about 23°C to a Superose 12 HR 10/30 HPLC column (Pharmacia) and proteins were eluted with 50mM bis-Tris/HCl, pH6.2 at a flow rate of 0.3ml/min. The active fractions were identified, pooled, and concentrated and transferred into 50mM sodium formate buffer, pH3.8 using a Centricon-30 microconcentrator. The enzyme solution in formate buffer was purified further using cation exchange chromatography. Samples of enzyme were applied in 2ml aliquots to a Mono S HR 5/5 FPLC column (Pharmacia) equilibrated in 50mM sodium formate (pH3.8) at about 23°C. The column was washed with the equilibration buffer (10ml) and the bound protein was eluted at 60ml/hr. using a 20ml linear gradient from 0mM to 430mM NaCl in the formate equilibration buffer. Phytase was purified by this method with a yield of 18.4% that had a specific activity of approximately 275,900 (PU/mg) with a calculated purification of 130-fold (Table A)

TABLE A

Summary of Purification of Phytase					
Step	Total Activity (PU)	Total Protein (mg)	Specific Activity (PU/mg)	Yield (%)	Purification (fold)
Culture filtrate	4,486,680	2,119	2,117	100	1
Ammonium sulphate supernatant	3,771,750	1,263	2,986	84.1	1.4
Octyl Sepharose	1,765,881	32.3	54,671	39.4	26
DEAE-Sepharose	1,453,470	8.4	173,032	32.4	82
Superose 12	1,010,888	57	177,349	22.5	84
Mono S	827,566	3.0	275,885	18.4	130

[0060] Acid phosphatase containing fractions from the pooled Octyl-Sepharose fraction, above, were first concentrated by ultrafiltration on Amicon PM10 filter, and then subjected to molecular-sieve chromatography on a 2.6cm x 94cm Sephacryl S-200 (Pharmacia) column equilibrated in 50mM bis-Tris/HCl (pH6.2). Proteins were eluted at 20ml/hr and fractions with activity were pooled and separated further by anion exchange chromatography on a 5cm x 7cm DEAE-Sepharose (Pharmacia) column equilibrated in 50mM bis-Tris/HCl, pH6.2. The column was washed with 100ml of equilibration buffer and adsorbed proteins were eluted using a 200ml linear gradient from 0.0M to 0.5M NaCl in equilibration buffer. Pooled active fractions were then concentrated; transferred to 20mM bis-Tris/HCl, pH6.0 by ultrafiltration on an Amicon PM10 membrane; and, subjected to a second step of anion exchange chromatography, this time using a Mono Q HR 5/5 HPLC column (Pharmacia) equilibrated in 20mM bis-Tris/HCl, pH6.0 at about 23°C. The sample was applied in 3.5ml aliquots; the column was washed with 10ml of the equilibration buffer; and, the proteins were eluted at 60ml/hr using a 20ml linear gradient from 0.0mM to 350mM NaCl in the equilibration buffer. Fractions containing enzyme activity were pooled, concentrated to a total volume of 400µl, and transferred into 20mM bis-Tris/HCl, pH6.2 containing 150mM NaCl using a Centricon-30 microconcentrator. Additional purification was accomplished, first by molecular-sieve chromatography on a Superose 12 HR 10/30 HPLC column (Pharmacia) equilibrated in the bis-Tris/HCl sample buffer. Aliquots of 100µl were applied to this column and proteins were eluted at 23°C at a rate of 18ml/hr. Fractions containing enzyme activity were pooled, transferred into 20mM histidine/HCl, pH5.8 buffer using a PD10 gel filtration column, and subjected to a second step of purification by anion exchange chromatography on a Mono Q HR 5/5 HPLC column. Aliquots of 1ml were applied to the column, the column was washed with 5ml of the histidine/HCl sample buffer at about 23°C. Proteins were eluted at a rate of 60ml/hr using a linear 20ml gradient from 0mM to 350mM NaCl in equilibration buffer. The pH2.5 acid phosphatase was purified by this method at 13% yield with a 126-fold purification, Table B.

TABLE B

Summary of Purification of pH2.5 Phosphatase					
Step	Total Activity (HFU)	Total Protein (mg)	Specific Activity (HFU/mg)	Yield (%)	Purification (fold)
Culture filtrate	116,523,000	2,119	54,990	100	1
Ammonium sulphate supernatant	88,275,000	1,263	69,893	75.8	1.3
Octyl Sepharose	68,296,470	583	117,147	58.6	2.1
Sephacryl	52,237,600	97.9	533,581	44.8	9.7
DEAE-Sepharose	46,127,692	54.6	844,830	39.6	15.4
Mono Q	19,326,753	3.28	5,892,303	16.6	107
Superose	16,876,978	nd	nd	14.5	nd
Mono Q	15,197,050	2.2	6,907,750	13.0	126
nd = not determined					

[0061] Phytase: Phytase as purified by the methods recited (above) exhibited an apparent molecular weight on SDS-PAGE of approximately 80,000-86,000 daltons. The protein as substantially purified (i.e., on SDS-PAGE) gave a

positive reaction with periodic acid Schiff staining for carbohydrate (i.e., the purified phytase enzyme is a glycoprotein). After treating the purified phytase with a mixture of endoglycosidase F/N-glycosidase F the apparent molecular weight of the enzyme (on SDS-PAGE) was shifted to about 45,000-48,000 daltons, suggesting that about 44% of the molecular mass may be attributable to carbohydrate. (The type of carbohydrate linkage and the nature of the moiety was not determined.) The molecular weight of the undenatured purified phytase enzyme was shown to be approximately 90,000 daltons by molecular-sieve gel filtration (i.e., based on its Stokes radius), and an apparent molecular weight of 100,000 daltons was observed by native gradient gel PAA electrophoresis. The latter results suggest that the undenatured purified phytase enzyme exists as a monomer. The isoelectric point of the subject phytase is 5.3 by isoelectric focusing.

[0062] pH2.5 acid phosphatase: pH2.5 acid phosphatase, purified by the methods recited above, exhibited an apparent subunit molecular weight on SDS-PAGE of 66,000 daltons. The substantially purified enzyme (i.e., on SDS-PAGE) gave a positive reaction with periodic acid Schiff staining indicating that pH2.5 acid phosphatase is also a glycoprotein. After removing carbohydrate with endoglycosidase F/N-glycosidase F the protein exhibited an apparent molecular weight of 46,000-49,000 daltons by SDS-PAGE. (The glycosidic linkage and nature of the carbohydrate moiety was not characterized.) pH2.5 acid phosphatase exhibits an apparent molecular weight of 280,000 daltons by native gradient PAA gel electrophoresis, suggesting that the purified undenatured enzyme exists as a tetramer of four 66,000-dalton subunits. The isoelectric point of acid phosphatase by isoelectric focusing is approximately 4 to 4.25.

Catalytic properties of the purified phytase and pH2.5 acid phosphatase enzymes:

[0063] Phytase: The purified phytase exhibited optimal enzyme activity (as measured at 37°C, in conditions of excess substrate), at a pH of approximately pH5.0 with a shoulder at pH2.5 and >20% of maximal enzyme activity in the range of pH2 to pH7 (FIGURE 1). The temperature optimum for the purified phytase enzyme (at optimal Na-phytate substrate and pH5.0) was found to be in the range of 55-60°C with >20% of maximal enzyme activity in the range of 25°C to 65°C (FIGURE 2). The purified phytase enzyme catalyzed hydrolysis of sodium phytate in the absence of added metal ion (i.e., the enzyme exhibited no absolute requirement for metal ions and thus was "metal ion independent"); however, the activity of the purified phytase enzyme was increased in the presence of Mn^{2+} , Mg^{2+} and Ca^{2+} .

[0064] pH2.5 acid phosphatase: The apparent K_m of the pH2.5 acid phosphatase for Na-phytate substrate at 37°C and pH2.5 was determined to be about 0.7mM; and, for paranitrophenylphosphate (PNP) at 37°C and pH2.5 the apparent K_m was 4mM. The purified pH2.5 acid phosphatase enzyme exhibited a pH optimum in the range of pH2.0 to pH2.5 (i.e., at 37°C, in the presence of optimal concentrations of Na-phytate substrate) with >20% of maximal activity in the range of pH1.5 to pH3.5 (FIGURE 3). The temperature optimum for pH2.5 acid phosphatase-mediated hydrolysis of Na-phytate was found to be about 55°C with >20% of maximal enzyme activity in the range of 25°C to 60°C (FIGURE 4). For comparison the pH/activity profile of a commercial non-purified Finase preparation is shown in FIGURE 5, and the temperature/activity profile is shown in FIGURE 6.

[0065] Degradation of phytate by phytase and pH2.5 acid phosphatase and mixtures thereof: Finase is a commercial preparation of enzymes from *Aspergillus* that includes a mixture of phytase and phosphatase. The ratio of pH2.5 acid phosphatase (HFU) activity to phytase activity (PU) of Finase was determined to be approximately 7:1. A comparison was made of the rate of phytate degradation by a commercial Finase enzyme preparation with the rate of the degradation by preparations of purified phytase, purified pH2.5 acid phosphatase and by mixtures thereof containing different acid phosphatase to phytase ratios. The ratio of acid phosphatase activity (HFU) to phytase activity (PU) of the purified phytase is approximately 0.4:1 whereas the acid phosphatase exhibits no phytase activity at pH5.0. The ratios of the enzyme mixtures are indicated in Tables 1.a and 1.b. The comparison was made at pH2.5 37°C and at pH5.0 37°C using 10mM Na-phytate as substrate. The enzyme activities of all different preparations used in the experiments at pH2.5 were 10,000HPU per mmol of Na-phytate substrate (HPU is the unit of phytase activity measured at pH2.5 using 0.2M glycine (HCl) buffer pH2.5 instead of Na-citrate buffer, see *Materials and Methods* at the end of Example 1). In the experiments conducted at pH5.0 the enzyme dosage per mmol of Na-phytate substrate was 10,000PU, with the exception of the experiment carried out using purified acid phosphatase alone. Samples were taken from the reaction mixture after the times indicated (hours, h; 0, 1, 2, 8, 24, and 48h); the samples were freeze-dried and then later analyzed for inositol hexa-, penta-, tetra- and tri- phosphates (i.e., IP6, IP5, IP4 and IP3, respectively), as well as for free inositol (Ins) and inorganic phosphorus (Pi). Inositol hexa-, penta-, tetra-, and triphosphates were analyzed by the method of Sandberg et al. (1987). Inositol was analyzed by HPLC using Sugar Pak 1 column (300mm x 6.5mm, Waters), 0.1mM Ca-EDTA as eluent (Calcium-Titriplex Merck 8439), flow 0.6ml/min at 90°C. The detection was by RI (Waters), using myo-inositol (Fluka) 0.1-0.5mg/ml as the standard. Inorganic phosphorus was analyzed as described in *Materials and Methods* in the end of Example 1. The results of these experiments are presented in Table 1a and Table 1b, below.

TABLE 1a

pH2.5		Yield, % of Theoretical ^a					
Enzyme(s) dosage/mmol substrate	Time (h)	IP6	IP5	IP4	IP3	Ins	P _i
Finase 10,000 HPU	0	100	0	0	0	0	103
	1	40	13	19	0	0	
	2	39	20	51	8	0	
	8	0	0	2	38	5	
	24	0	0	0	0	57	
	48	0	0	0	0	94	
Phytase 10.000 HPU	0	100	0	0	0	0	87
	1	20	29	20	0	0	
	2	5	30	68	22	0	
	8	0	0	(+)	24	0	
	24	0	0	0	0	0	
	48	0	0	0	0	0	
pH2.5 Acid Phosphatase 10,000 HPU	0	100	0	0	0	0	88
	1	67	8	26	0	0	
	2	54	9	37	(+)	0	
	8	26	2	42	12	1	
	24	2	1	8	4	40	
	48	1	0	5	1	67	
Phytase 9,000 HPU pH2.5 Acid Phosphatase 1,000 HPU HFU:PU 0.8:1	0	100	0	0	0	0	0
	1	44	23	32	20	0	16
	2	6	13	71	17	0	31
	8	0	2	10	0	2	79
	24	0	0	0	0	33	93
	48	0	0	0	0	63	98
Phytase 5,000 HPU pH2.5 Acid Phosphatase 5,000 HPU HFU:PU	0	100	0	0	0	0	0
	1	38	12	47	(+)	0	15
	2	17	7	76	(+)	6	27
	8	0	0	7	19	12	69
	24	0	0	0	0	76	99

a. Yield, % of theoretical: the starting concentration of 10mM Na-phytate substrate (IP6) in the assays was considered equal to 100% theoretical yield (i.e., of IP6); for IP6, the concentration of residual Na-phytate at the end of the assay was divided by the starting concentration to obtain the % indicated in Tables 1a and 1b; for IP5, IP4, IP3, Ins, and P, the maximal theoretical concentration of each degradation product that could be released from IP6 (i.e., by acid hydrolysis) was calculated and considered equal to 100% theoretical yield of the respective degradation product; the concentration of residual degradation product at the end of the assay was divided by the theoretical concentration to obtain the % indicated in Tables 1a and 1b; (+) traces.

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TABLE 1a (continued)

pH2.5		Yield, % of Theoretical ^a					
Enzyme(s) dosage/mmol substrate	Time (h)	IP6	IP5	IP4	IP3	Ins	P _i
4.4:1	48	0	0	0	0	101	104
Phytase 1,000 HPU pH2.5 Acid Phosphatase 9,000 HPU	0	100	0	0	0	0	0
	1	40	16	39	(+)	0	10
	2	27	8	26	(+)	1	17
	8	7	2	21	17	9	45
	24	0	0	6	5	46	79
HFU:PU 36.4:1	48	0	0	0	0	102	103

a. Yield, % of theoretical: the starting concentration of 10mM Na-phytate substrate (IP6) in the assays was considered equal to 100% theoretical yield (i.e., of IP6); for IP6, the concentration of residual Na-phytate at the end of the assay was divided by the starting concentration to obtain the % indicated in Tables 1a and 1b; for IP5, IP4, IP3, Ins, and P, the maximal theoretical concentration of each degradation product that could be released from IP6 (i.e., by acid hydrolysis) was calculated and considered equal to 100% theoretical yield of the respective degradation product; the concentration of residual degradation product at the end of the assay was divided by the theoretical concentration to obtain the % indicated in Tables 1a and 1b; (+) traces.

TABLE 1b

pH5.0		Yield, % of Theoretical ^a					
Enzyme(s) dosage/mmol substrate	Time (h)	IP6	IP5	IP4	IP3	Ins	P _i
Finase 10,000 PU	0	100	0	0	0	0	88
	1	51	16	24	0	0	
	2	35	22	29	14	0	
	8	0	7	1	18	0	
	24	0	0	0	0	18	
	48	0	0	0	0	52	
Phytase 10,000 PU	0	100	0	0	0	0	90
	1	43	33	27	11	0	
	2	23	18	24	21	0	
	8	1	2	6	24	0	
	24	0	0	0	0	0	
	48	0	0	0	0	0	
pH2.5 Acid Phosphatase 0 PU 10,000 HPU	0	100	0	0	0	0	0
	1	107	0	0	0	0	0
	2	106	0	0	0	0	0
	8	105	0	0	0	0	0
	24	93	0	(+)	(+)	0	1
	48	96	0	(+)	(+)	0	1
Phytase 10,000 PU pF2.5 Acid Phosphatase 1,000 HPU	0	100	0	0	0	0	0
	1	30	26	45	(+)	0	18
	2	27	23	36	32	0	21

a. As in Table 1a, above.

TABLE 1b (continued)

pH5.0		Yield, % of Theoretical ^a					
Enzyme(s) dosage/mmol substrate	Time (h)	IP6	IP5	IP4	IP3	Ins	P _i
HFU:PU 1.4:1	8	1	2	10	29	0	46
	24	0	0	(+)	(+)	4	82
	48	0	0	(+)	(+)	17	90
Phytase 10,000 PU pH2.5 Acid Phosphatase 5,000 HPU HFU:PU 5.4:1	0	100	0	0	0	0	0
	1	34	12	65	0	0	21
	2	27	22	38	28	0	24
	8	1	2	9	27	0	50
	24	0	0	0	0	21	88
	48	0	0	0	0	59	96
Phytase 10,000 PU pH2.5 Acid Phosphatase 9,000 HPU HFU:PU 9.4:1	0	100	0	0	0	0	0
	30	30	11	54	0	0	24
	2	31	22	36	28	1	24
	8	(+)	2	9	25	9	51
	24	0	0	0	0	46	88
	48	0	0	0	0	102	102

a. As in Table 1a, above.

[0066] The results presented in Tables 1a and 1b show that the purified phytase and pH2.5 acid phosphatase enzymes and the Finase commercial enzyme mixture catalyzed hydrolysis of Na-phytate to inositol and inorganic phosphate, but at differing rates and with different pH optima. Under optimal conditions of pH for each of the purified enzymes (i.e., pH5.0 for purified phytase; and pH2.5 for the purified pH2.5 acid phosphatase) approximately equivalent amounts of inorganic phosphate were released in 48 hours by all three enzyme preparations (i.e., 90% P_i for phytase; 88% P_i for acid phosphatase; and, 88% or 103% for the Finase mixture). However, the results also show that inositol (Ins, Tables 1a and 1b) production is markedly slower at pH5.0 than at pH2.5. Inositol, as the end product of Na-phytate hydrolysis, could not be detected at pH2.5 nor at pH5.0 when purified phytase enzyme is used alone, although IP6, IP5, IP4 and IP3 were completely removed during the hydrolysis time. It can be concluded that the probable hydrolytic end products of the purified phytase enzymes are inositol di-and/or monophosphates. In contrast, purified acid phosphatase catalyzed degradation of phytate (i.e., IP6 = inositolhexaphosphates) at pH2.5, producing inositol as an end product. These combined results suggested that in a commercial mixture of phosphatases, such as Finase, phytase alone is not sufficient to enable complete conversion of phytate to inositol and inorganic phosphate. Rather, the results suggested that inositol end products may be derived from the action of enzymes with substrate specificity similar to that of purified pH2.5 acid phosphatase. The possibility was therefore considered that the products of phytate hydrolysis by phytase (i.e., IP5, IP4, IP3, IP2, IP1) might serve as useful substrates for acid phosphatase that would convert the inositolphosphates into free inositol and inorganic phosphate. The results thus suggested a previously unsuspected cooperative enzyme activity for phytate degradation between phytase and pH2.5 acid phosphatase. To further explore this possibility the substrate specificities of the purified phytase and pH2.5 acid phosphatase were evaluated.

[0067] The substrate specificities of purified phytase and pH2.5 acid phosphatase were compared at 37°C using equivalent concentrations of phytate substrate (i.e., 20mM) and temperature (i.e., 37°C). Phytase activity was measured at pH5.0 and acid phosphatase activity was measured at pH2.5 using the molybdate phosphate detection system described in the *Materials and Methods* section appearing at the end of EXAMPLE 1. A comparison of the relative activities of the two different enzymes on different substrates is presented in Table 2, below.

TABLE 2

Substrate Specificity of Substantially Purified Phytase and pH2.5 Acid Phosphatase		
Substrate	Phytase Relative Activity(%) ^a	pH2.5 Acid Phosphatase Relative Activity(%) ^b
Phytic acid Na-salt	100	36
Phytic acid K-Mg-salt	106	34
Phytic acid Ca-salt	56	43
Glucose 6-phosphate	11	62
ATP	7	72
Fructose 1,6-diphosphate	8	107
Fructose 6-phosphate	1	18
L-a-glycerophosphate	1	71
p-nitrophenyl phosphate	24	100

a.) Percent relative activity = "the measured activity calculated as a % of that obtained with a standard substrate of phytase (i.e., Na-phytate defined as 100%)."

b.) Percent relative activity = "the measured activity calculated as a % of that obtained with a standard substrate of acid phosphatase (i.e., p-nitrophenyl phosphate; defined as 100%)"

[0068] The results presented in Table 1a, Table 1b and Table 2 show that purified phytase, while effective in mediating the hydrolysis of phytic acid, was less effective in catalyzing hydrolysis of other simpler phosphate-containing substrates. In contrast, purified pH2.5 acid phosphatase, while relatively effective in hydrolyzing simpler phosphates, was relatively ineffective in catalyzing hydrolysis of phytic acid. The results support the notion of a cooperative enzymatic mixture wherein the products of a purified phytase (e.g., inositol di- and mono-phosphates) might serve as substrates for a purified pH2.5 acid phosphatase so that free inositol and inorganic phosphate are the ultimate products of the reaction.

"Balanced" enzyme mixtures:

[0069] The possibility was next considered that an effective cooperative enzymatic mixture might be constructed by optimizing the amounts of purified phytase and purified pH2.5 acid phosphatases that were mixed together in a preparation so that the activity of the two different enzymes was "balanced", i.e., to achieve cooperative enzyme activity at a particular pH and temperature to give the optimal rate, efficiency, and completeness of phytate degradation to free inositol and inorganic phosphate. Studies were conducted in which mixtures of purified phytase and pH2.5 acid phosphatase were formulated at defined ratios of the two enzyme activities, but while such "balanced" mixtures of purified phytase and pH2.5 acid phosphatase offered advantages of increased rate of phytate hydrolysis and more complete conversion to free inositol and inorganic phosphate over commercial preparations, it was not considered cost-effective to purify, standardize, quality control (QC) and quality assure (QA) such a "balanced" mixture of enzymes for use in most commercial processes (e.g., for animal feeds) within desired ranges of ratios of enzyme activities, e.g., pH2.5 acid phosphatase to phytase.

[0070] To solve these problems the possibility was next considered that molecular techniques might be used to produce recombinant enzymes that were of sufficiently high quality to provide uniform standardized preparations, and with the necessary cost-efficiency and QC/QA properties required for commercial products of "balanced" mixtures.

Amino acid sequencing of phytase and pH2.5 acid phosphatase:

[0071] In order to obtain internal amino acid sequences of the purified proteins, peptide fragments were prepared using TPCK-trypsin (as described, see *Materials and Methods*, below). In addition, purified pH2.5 acid phosphatase was alkylated (i.e., using 4-vinyl pyridine) and then digested with lysylendopeptidase C, as described (*Materials and Methods*). The resultant peptides were purified by reverse-phase high pressure liquid chromatography (RP-HPLC) on a C18 RP column (as described in the *Materials and Methods* section, below). Amino terminal sequencing of purified peptides was conducted using a gas-pulsed liquid phase sequencer, and the released PTH amino acids were analyzed by RP-HPLC (as described, *Materials and Methods*). Carboxy-terminal sequencing was conducted using kinetic carboxypeptidase Y digestion, PITC derivatization, and RP-HPLC analysis of the PITC amino acids.

[0072] The amino acid sequences of the tryptic peptides of phytase and pH2.5 phosphatase, as well as the lysylendopeptidase peptides of pH2.5 phosphatase are presented in Tables 3 and 4, below. As the results presented in Table 3 and 4 indicate, some peptide preparations were not pure. Tables 3 and 4 also present a comparison of the peptide amino acid sequences with the amino acid sequence deduced from nucleotide sequencing of the genes and the cDNA (Example 2, below), i.e., brackets [] in Tables 3-4.

[0073] The amino terminal sequence of purified *Aspergillus niger* var. *awamori* strain ALKO243 phytase showed N-terminal sequence (i.e., peptide #1081/N-phy Table 3; LAVPAS(R)NQSTXDT) similar to, but not identical with the reported amino terminal sequence in an *A. ficuum* phytase (i.e., Ullah, 1988; LAVPASRNQSSGDT). One peptide (i.e., #420/10phy; LYVEMMQ(N)QA(E)Q(T)PLV) was similar to, but not identical in sequence to, a reported internal peptide in an *A. ficuum* phytase (i.e., Ullah, 1988, MMQCQAEQEPLVRVLVNDRX). Carboxyterminal sequencing of phytase gave the sequence XSA-OH. One peptide (i.e., #675; Table 3) contained a KDPR sequence homologous with sequence reportedly present in other phosphatases (namely KDPR; Ullah, 1991).

TABLE 3

Amino acid sequence of isolated peptides of phytase

Peptide Nos.	Amino Acid Sequence ^a	[Amino Acid Sequence Deduced from DNA sequence] ^b
#132/12 phy	Tyr-Tyr-Gly-His(Leu)-Gly-Ala-Gly-Asn-Pro-Leu-Gly-Pro-Thr-Gln [Tyr-Tyr-Gly- His---Gly-Ala-Gly-Asn-Pro-Leu-Gly-Pro-Thr-Gln]	
#133	Thr-Gly-Tyr-Val-Gln(Asn)-Tyr-Val-Gln-Met-(Gln)	[not found in DNA]
#242/1 phy	Ala-Gln-Pro-Gly-Gln-Ala-Ala-Pro-Lys	[Ala-Gln-Pro-Gly-Gln-Ser-Ser-Pro-Lys]
#420/10 phy	Leu-Tyr-Val-Glu-Met-Met-Gln-(Asn)-Gln-Ala-(Glu)-Gln-(Thr)-Pro-Leu-Val [Leu-Tyr-Val-Glu-Met-Met-Gln- Cys -Gln-Ala- Glu -Gln- Glu -Pro-Leu-Val]	
#410/13 phy	Phe-Ile-Glu-Gly-Phe-Gln-Ser-Asp-Lys	[Phe-Ile-Glu-Gly-Phe-Gln-Ser-Asp-Lys]
#416/7 phy	Tyr-Ala-Phe-Leu-Lys	[Tyr-Ala-Phe-Leu-Lys]
#659/6 phy	Gly-Leu-Ser-Phe-Ala-Arg	[Gly-Leu-Ser-Phe-Ala-Arg]
#670̜/2 phy	Val-Ile-Ala-Ser-Gly-Glu-Lys	[Val-Ile-Ala-Ser-Gly-Glu-Lys]
#418/3 phy	Phe-Tyr-Gln-Arg	[Phe-Tyr-Gln-Arg]
#785/11 phy (not pure)	Phe-Tyr-Gln-Arg {= #418, above} and Asp-Ser-Phe-Val-Arg [Asp-Ser-Phe-Val-Arg]	
#248 (not pure)	Val/Tyr-Leu/Glu-Val/Ser-Asn/Leu-Asp/Gln	[not possible to compare to DNA]
#784/9 phy	Tyr-Glu-Ser-Leu-Thr-Arg	[Tyr-Glu-Ser-Leu-Thr-Arg]
#675 (not pure)	Ser-Ala-Ala-Ser-Leu-Asn-Ser {a fragment of the trypsin enzyme} Leu-Lys-Asp-Pro-Arg	[Leu-Lys-Asp-Pro-Arg]
#783 (not pure)/ 4 phy	Val-Ile-Ala-Ser-Gly-Glu-Lys {small amount = #670 and 796, above} Tyr-Pro-Thr-Glu-Ser-Lys	[Tyr-Pro-Thr-Glu-Ser-Lys]
#244 (not pure)	Tyr/Asp-Phe/Pro-Asn/Ala-X/X-Gly	[not possible to compare to DNA]
#793	Leu-Glu-Asn/Pro-Asp/Phe-Leu-Asp/Ser-Gly/Leu-Phe/Val-Thr-Leu) [Leu-Glu- Asn - Asp -Leu- Ser - Gly - Val -Thr-Leu- Thr]	
#792 (double sequence)/15 phy	Tyr-Tyr-Gly-His-Gly-Ala-Gly-Asn-Pro-Leu-Gly-Pro-Thr-Gln-Gly-Val-Gly-Tyr-Ala- Asn-Glu-Leu-Ile-Ala {= #132 (half of above) and Val-Tyr-Phe-Ala-Gln-Val-Leu-Ser From this double sequence, the following sequences can be deduced Val-Thr-Phe-Ala-Gln-Val-Leu-Ser [Val-Thr-Phe-Ala-Gln-Val-Leu-Ser] and Tyr-Tyr-Gly-His-Gly-Ala-Gly-Asn-Pro-Leu-Gly-Pro-Thr-Gln-Gly-Val-Gly-Tyr- Ala-Asn-Glu-Leu-Ile-Ala [Tyr-Tyr-Gly-His-Gly-Ala-Gly-Asn-Pro-Leu-Gly-Pro-Thr-Gln-Gly-Val-Gly-Tyr-Ala- Asn-Glu-Leu-Ile-Ala	
#800/13 phy	Phe-Ile-Glu-Gly-Phe-Gln-Ser-Thr	[Phe-Ile-Glu-Gly-Phe-Gln-Ser-Thr]

TABLE 3 (continued)

Peptide Nos.	Amino Acid Sequence ^a	[Amino Acid Sequence Deduced from DNA sequence] ^b
#797/14 phy	Asp/Asn-Tyr-Leu-Gln-Ser-Leu-Lys	[Asp -Tyr-Leu-Gln-Ser-Leu-Lys]
#795 (Odd behavior in peptide sequencing)	Asn-Ile-Glu-Pro-Phe-Gln-Val-Asn [not found in DNA sequence.]	
#799/8 phy	Val-Leu-Val-Asn-Asp-Arg {≈ #248, above}	[Val-Leu-Val-Asn-Asp-Arg]
#1081 /N-phy	Leu-Ala-Val-Pro-Ala-Ser-(Arg)-Asp-Gln-Ser-Thr- X -Asp-Thr [Leu-Ala-Val-Pro-Ala-Ser- Arg -Asn-Gln-Ser-Thr-Cys-Asp-Thr]	
C-terminal/C phy	-(Arg)-Ser-Ala-OH	[Cys -Ser-Ala-End]

a.) peptide sequence, X = amino acid not detected; / = either one or the other of the two indicated amino acids may be present, the assay was not definitive, () = the presence of the amino acids in parentheses is subject to question because of a weak signal of the PTH-amino acid; phytase peptides designated (phy) were obtained by tryptic digestion; b.) [] = [peptide sequence deduced from DNA sequence, Example 2, below]; and, #: peptides number.

[0074] In sequencing of pH2.5 phosphatase, no results were obtained from amino terminal sequencing of either native or alkylated proteins purified from *Aspergillus niger* var. *awamori* strain ALKO243. One peptide (i.e., #1107T17Lpho, Table 4, below) yielded a sequence that was identical to an amino terminal sequence reported in a phosphatase purified from *A. ficuum* (i.e., Ullah & Cummins, 1987; FSYGAAIPQSTQEKQFSQEFRDG). Peptide #941C/10Lpho from purified pH2.5 phosphatase (ALKO243) may be a continuation of the #1107/7Lpho sequence, since it appears to be included in the *A. ficuum* N-terminus. The peptide #1112T/3Tpho seems to be a continuation of peptide #943C/11Lpho, since it appears to have a partially overlapping sequence (i.e., FSSG in #1112T/3Tpho). The pH2.5 phosphatase peptide #816C/1Lpho contains a possible active site consensus sequence (i.e., RHGX RXP)

TABLE 4

Amino Acid Sequence of Isolated Tryptic (T) and Lysylendopeptidase (C) Peptides^a
of pH2.5 Phosphatase

Peptide Nos.	Amino acid sequencing ^b [DNA sequencing] ^c
#816C/1 Lpho	Arg-His-Gly-Glu-Arg-Tyr-Pro-Ser-Pro-Ser-Ala-Gly-Lys [Arg-His-Gly-Glu-Arg-Tyr-Pro-Ser-Pro-Ser-Ala-Gly-Lys]
#817C and #1107T/7 Lpho	Phe-Ser-Tyr-Gly-Ala-Ala-Ile-Pro-Gln-Ser-Thr-Gln-Glu-Lys [Phe-Ser-Tyr-Gly-Ala-Ala-Ile-Pro-Gln-Ser-Thr-Gln-Glu-Lys]
#847C/5 Lpho	Asp-Ile-Glu-Glu Ala-Leu-Ala-Lys [Asp-Ile-Glu-Glu-Ala-Leu-Ala-Lys]

TABLE 4 (continued)

Peptide Nos.	Amino acid sequencing ^b [DNA sequencing] ^c
#826C (not pure)	Ser/Ala-Ile-Glu/Pro-(Glu) [not possible to compare to DNA]
#943C/11 Lpho	Ala-Arg-Tyr-Gly-His-Leu-Trp-Asn-Gly-Glu-Thr-Val-Val-Pro-Phe-Phe-Ser-Ser-Gly [Ala-Arg-Tyr-Gly-His-Leu-Trp-Asn-Gly-Glu-Thr-Val-Val-Pro-Phe-Phe-Ser-Ser-Gly]
#938C (not pure)/2 Lpho	(Ser/Arg)-Tyr/His-Gly-Gly/Glu-Asn/Arg-Gly/Tyr-Pro-Tyr/Ser-(Pro)-Glu/Ser-(Ala)-(Gly) [only part of sequence compared to DNA sequence] [Tyr -Gly- Gly - Asn - Gly -Pro- Tyr]
#941C (not pure)/10 Lpho	Gln-Phe-Ser-Gln-Glu-Phe- X -Asp-Gly-Tyr-(Arg) {Predominant species} [Gln-Phe-Ser-Gln-Glu-Phe-Arg-Asp-Gly-Tyr] (Thr/His)-Tyr-Gly-Gly-Asn-Gly-X-Tyr/Pro (= #938C, above) {Minor species}
#1106T and #1112T/3 Tpho #1108T (not pure)/9 Tpho	Phe-Ser-Ser-Gly-Tyr-Gly-Arg [Phe-Ser-Ser-Gly-Tyr-Gly-Arg] Val-Ala-Phe-Gly-Asn-Pro-(Tyr) and (Asp/Glu)-Leu-Asn-Ala-Ile-Leu-Phe/Lys -from the sequence above the following sequences were deduced: Val-Ala-Phe-Gly-Asn-Pro-(Tyr) and (Asp/Glu)-Leu-Asn-Ala-Ile-Leu-Phe/Lys [Val-Ala-Phe-Gly-Asn-Pro- Tyr] and [not found in DNA sequence]
#1110T (not pure)/6 Tpho	Asp-Ile-Glu-Glu-Ala-Leu-Ala-Lys {=#847C, above} and Gln-Leu-Pro-Gln-Phe-Lys [Gln-Leu-Pro-Gln-Phe-Lys]
#1111T/4 Tpho	Val-Ser-Tyr-Gly-Ile-Ala [Val-Ser-Tyr-Gly-Ile-Ala]

a.) T: = trypsin digestion; C: = lysylendopeptidase C digestion of 4-vinylpyridine treated pH2.5 phosphatase;
b.) peptide sequence, X = amino acid not detected; / = either one or the other of the two indicated amino acids may be present, the assay was not definitive, () = the presence of the amino acids in parenthesis is subject to question because of a weak signal of the PTH-amino acid; phosphatase peptides (pho) obtained by lysylendopeptidase digestion are identified (L), alkylated phosphatase peptides obtained by trypsin digestion are identified (T); and, c.) [] = [peptide sequence deduced from DNA sequence, Example 2, below]; and, # : peptide number.

[0075] From these amino acid sequences one peptide sequence from phytase (i.e., #420, Leu Tyr Val Glu Met Met Gln (Asn) Gln Ala (Glu) Gln (Thr) Pro Leu Val with the questionable (Asn) corrected to Cys; Ullah, 1988; Table 3) and two from pH2.5 phosphatase (i.e., #81bC; Arg His Gly Glu Arg Tyr Pro Ser Pro Ser Ala Gly Lys and #11 10T; Gln Leu Pro Gln Phe Lys, Table 4) were selected as being useful for the preparation of degenerate oligonucleotide probes for molecular cloning, as described below in Example 2.

Materials and Methods:

Phytase and pH2.5 acid phosphatase enzyme assays: Molybdate detection system:

[0076] Both assays measure the amount of inorganic phosphate that is released by enzyme action as colorimetrically

quantifying using reduction of a phosphomolybdate complex. One phytase unit (PU) is the amount of enzyme which liberates, under the conditions described below, 1nmol of inorganic phosphate from Na-phytate in one minute at 37°C. One pH2.5 acid phosphatase unit (HFU) is the amount of enzyme which liberates, under the conditions indicated below, 1nmol of inorganic phosphate from P-nitrophenyl phosphate in one minute.

[0077] Phytase activity was determined by adding 1 ml of substrate (1% sodium phytate freshly prepared daily [Sigma #P3168], in 0.2M citrate buffer, pH5.0) to 1ml of diluted enzyme supernatant to initiate the hydrolysis of orthophosphate. After exactly fifteen minutes at 37°C, the hydrolysis reaction was terminated by the addition 2ml of 15% TCA (trichloroacetic acid, Merck #807) followed by mixing, cooling, and centrifugation to remove any precipitate that forms. Released orthophosphate was measured by the addition of an equal volume of freshly prepared Reagent C (i.e., 3 volumes of 1M sulfuric acid mixed with 1 volume of 2.5% (w/v) ammonium molybdate (Merck #1182) and 1 volume of 10% (w/v) ascorbic acid (Merck #127) to a 1:10 dilution of the hydrolysis reaction mixture (above). The Reagent C mixture was incubated at 50°C for twenty minutes. The absorbances were measured at 820nm against a reagent blank and known standards (1:100 - 1:400 dilutions of 9.0mM KH_2PO_4 ; Merck #4873) that were used to construct a standard curve. The amount of phosphate liberated by phytase was used to calculate phytase in the following manner: namely, the $A_{820\text{nm}}$ value for the phytase was compared with the $A_{820\text{nm}}$ values of the phosphate standard curve and after correcting for any dilution factors the phytase activity was obtained by dividing the phosphorus concentration (nmol/ml) by the hydrolysis time (i.e., 15 min).

[0078] pH2.5 acid phosphatase activity was determined in a similar fashion: namely, 0.1ml of diluted enzyme (diluted in 0.2M Glycine-HCl buffer, pH2.5) was added to 1.9ml of substrate (30mM p-nitrophenyl phosphate [Boehringer Mannheim] dissolved in 0.2M Glycine -HCl buffer) pH2.5. After a fifteen-minute incubation at 37°C, the reaction was terminated by addition of an equal volume of 15% TCA (as above). The released orthophosphate was measured using Reagent C (as described above), and the activity determined by a comparison to known diluted phosphate standards (as above) and by using the calculations described above.

Preparation of tryptic peptide fragments for amino acid sequencing:

[0079] Purified phytase (70µg) in 50mM Tris-HCl pH7.9 was digested with 2% (w/w) trypsin (TPCK-treated, Sigma) for 2 h at 37°C and then with a further 2% (w/w) trypsin for 21 h. Purified pH2.5 acid phosphatase in 100mM Tris-HCl pH8.0 was treated with 2% (w/w) trypsin for 20 h at 37°C and then with a further 2% (w/w) trypsin for 6 h. The peptides were purified as described below.

Preparation of lysylendopeptidase C peptide fragments for amino acid sequencing:

[0080] Purified pH2.5 acid phosphatase was alkylated using 4-vinylpyridine as follows: To lyophilized pH2.5 acid phosphatase (75µg) was added 40µl 0.5M Tris-HCl pH7.5 containing 6M guanidium hydrochloride, 2mM EDTA and 34mM DTT. After addition of 1µl 4-vinylpyridine (Sigma), the reaction mixture was kept at room temperature (22°C) for 1 h. The reaction was stopped by addition of 10ml 1.4M DTT. Alkylated phosphatase was then purified on HPLC with a C-1 reverse-phase column (TSK TMS 250; 0.46 x 4 cm) using a 20% to 70% ACN/0.06% TFA gradient (80% to 30% 0.1% TFA) in 30 min. The fractions absorbing at 218nm were pooled and evaporated in a Speed-Vac vacuum centrifuge. The dried sample was then resuspended by adding 60µl 70mM Tris-HCl pH9.1 and digested with 2% (w/w) lysylendopeptidase C (Wako Chemicals) for 2 h at 37°C. After addition of a further 2% (w/w) lysylendopeptidase C, the incubation at 37°C was prolonged to 26 h. The peptides were purified as described below.

Peptide purification and amino terminal sequencing:

[0081] The peptides obtained from enzymatic digestion (above) were separated by HPLC on a C-18 reverse-phase column (Vydac 218 TP B5; 0.46 x 25 cm) with a 90 min gradient from 0 to 60% ACN/0.06% TFA (100 to 40% 0.1% TFA). Absorbance at 218nm was used for detection of peptides. Amino terminal sequencing of the purified peptides, as well as the native proteins, was done by degrading them in a gas-pulsed liquid-phase sequencer (Kalkinen & Tilgmann, 1988). The released PTH-amino acids were analyzed on-line by using narrow-bore reverse-phase HPLC.

Carboxy terminal sequencing of phytase:

[0082] A lot of purified phytase (53µg) was digested with carboxypeptidase Y (Sigma, 0.6 U) in 50mM sodium acetate pH5.6 containing 10% urea and 0.05% SDS at room temperature (22°C). Samples of the digestion were withdrawn at various time points. These were dried in a Speed-Vac vacuum centrifuge and derivatized with phenylisothiocyanate (PITC) according to the amino acid analyzing kit Pico-Tag (Waters association). Analysis of the derivatized amino acids was performed by reverse-phase HPLC with the Pico-Tag C-18 column, and quantified by identically derivatized amino

acid standards.

EXAMPLE 2

Isolation and characterization of phosphatase genes: phytase and pH2.5 acid phosphatase

[0083] [General materials and methods are described in the section entitled "*Materials and Methods*," which follow the example.]

[0084] For molecular cloning of the phytase and the pH2.5 acid phosphatase genes interior peptides from the enzymes (as described above) were prepared from *A. niger* var. *awamori* strain ALKO243. The genomic DNA encoding phytase and pH2.5 acid phosphatase were cloned and cDNA was also cloned so that the complete coding sequence could be determined. Finally, expression vectors were constructed (as described in Example 3, below) that secreted each of the enzymes in a functional form, and a dual-gene-transformed strain was also selected that synthesized and secreted the desired cost-effective and "balanced" mixture of enzymes at a ratio of pH2.5 acid phosphatase to phytase centering upon the mixture the property of cooperative enzyme activity that is desirable in a particular commercial use, e.g., in animal feeds.

Design of oligonucleotide probes:

[0085] Several internal peptide fragments were sequenced from purified phytase and pH2.5 acid phosphatase, Example 1, above. A degenerate oligonucleotide complementary to all eight possible codon combinations of a chosen region (brackets, Table 3) of an internal phytase peptide was synthesized using the Pharmacia Gene Assembler Plus: namely, the peptide from phytase with the sequence Leu Tyr Val Glu Met Met Gln Cys Gln Ala Glu Gln (i.e., peptide #420, Table 3 with the questionable (Asn) corrected with Cys; Ullah, 1988). PHY-1, shown in Table 5, is a 17-base oligonucleotide mixture which contains one perfect match out of eight combinations.

[0086] A 17mer degenerate oligonucleotide, PHY-31, was designed from an acid phosphatase peptide: namely, peptide #816C from pH2.5 phosphatase (i.e., Arg His Gly Glu Arg Tyr Pro Ser Pro Ser Ala Gly Lys). Through the incorporation of a neutral inosine, one perfect match out of 64 possible combinations exists in PHY-31. The nucleotide sequence of oligo PHY-31 and corresponding peptide sequence is shown in Table 5. PHY-34 is a 17mer mixture with 128-fold degeneracy constructed to coding sequence for peptide #11 10T of pH2.5 acid phosphatase; PHY-35 is a 17mer mixture with 64-fold degeneracy also constructed to coding sequence for peptide #1110T. Both PHY-34 and PHY-35 are necessary for complete representation of Peptide #1110T. Peptide #1110T is derived from a trypsin digestion of purified native pH2.5 acid phosphatase (Table 4, above).

TABLE 5
Oligonucleotide Probes for Phytase and pH2.5 Phosphatase

Enzyme	Amino Acid Sequence ^a	Oligonucleotide Probe
Phytase	Peptide #420 (SEQ. ID. NO. 1): Leu Tyr Val Glu [Met Met Gln Cys Gln] Ala Glu Gln	Oligo PHY-1: 3' - CAT CAT GTT ACG GTT CG -5' (SEQ. ID. NO. 4) 3' - CAT CAT GTC ACA GTC CG -5' (SEQ. ID. NO. 5)
	pH2.5 Acid P ^h ase Peptide #816C (SEQ. ID. NO. 2): Arg [His Gly Glu Arg Tyr Pro] Ser Pro Ser Ala Gly Lys	Oligo PHY-31: 3' - GTG CCG CTC GCI ATG GG -5' (SEQ. ID. NO. 6) 3' - GTA CCA CTT TCI ATA GG -5' (SEQ. ID. NO. 7) 3' - GTG CCG CTC GCI ATG GG -5' (SEQ. ID. NO. 8) 3' - GTG CCC CTC GCI ATG GG -5' (SEQ. ID. NO. 9)
	Peptide # 1110T (SEQ. ID. NO. 3) [Gln Leu Pro Gln Phe Lys]	Oligo PHY 34: 5' - CAA CTG CCG CAA TTT AA -3' (SEQ. ID. NO. 10) 5' - CAG CTA CCA CAG TTC AA -3' (SEQ. ID. NO. 11) 5' - CAA CTT CCT CAA TTT AA -3' (SEQ. ID. NO. 12) 5' - CAA CTC CCC CAA TTT AA -3' (SEQ. ID. NO. 13)
		Oligo PHY 35: 5' - CAA TTA CCG CAA TTT AA -3' (SEQ. ID. NO. 14) 5' - CAG TTG CCA CAG TTC AA -3' (SEQ. ID. NO. 15) 5' - CAA TTA CCT CAA TTT AA -3' (SEQ. ID. NO. 16) 5' - CAA TTA CCC CAA TTT AA -3' (SEQ. ID. NO. 17)

a.) | | = region of sequence utilized in construction of degenerate oligonucleotides; peptides as described in Tables 3 and 4, above.

Probing genomic ALKO243 DNA with degenerate oligonucleotides:

[0087] In order to evaluate the specificity of the degenerate oligonucleotides, total genomic DNA from ALKO243 was probed with PHY-1 end labelled with [γ -³²P]-ATP to a high specific activity using *E. coli* Polynucleotide T4 kinase [BRL]. FIGURE 7 shows that with a high stringency wash, a unique band hybridized to phytase oligo PHY-1. At this stringency, a single band was recognized by the degenerate oligo which made it a good candidate for library screening.

FIGURE 7. *A. niger* var. *awamori* strain ALKO243 genomic DNA probed with phytase oligonucleotide PHY-1. Genomic DNA was isolated by a neutral lysis method. Briefly, finely ground frozen dried mycelia was lysed with a 4% SDS-TE buffer Cell debris was removed and supernatant was removed and extracted twice with an equal volume of Tris-saturated phenol:chloroform (1:1). Genomic DNA was precipitated with NH₄OAc and EtOH. Pelleted DNA was purified by ultracentrifugation through CsCl as recovered as described by Maniatis et al. Hybridization to genomic DNA with (γ ³²P) ATP labelled degenerate oligos (Maniatis et al., 1982) was done at 42°C overnight on filters in oligo hybridization solution (6X SSPE, 0.5% SDS, 3X Denhardt's, 100µg/ml tRNA). Non-specific binding was removed by washing the filters twice for 30 minutes at room temperature with 2XSSC, 0.1% SDS, and once for 5 minutes at 42°C in fresh solution. Overnight exposure on Kodak X-Omat AR film with intensifying screens revealed positively hybridizing bands.

[0088] Likewise, genomic DNA from ALKO243 was probed with radiolabelled specific pH2.5 acid phosphatase oligo PHY-31 (under the conditions described in FIGURE 7, above). The autoradiogram is shown in FIGURE 8. Although the specificity of PHY-31 was not as high as with PHY-1, it appeared as if, at most, two bands were recognized by this oligonucleotide probe.

Isolation and characterization of the phytase gene:

[0089] Genomic DNA was partially digested with Sau3A in order to produce fragments 10-23 kb in length. Digested DNA was ligated to BamHI cut dephosphorylated Lambda Dash II vector arms [Stratagene]. The ligation was packaged *in vitro* using Stratagene's Gigapack Gold packaging extracts. Packaged phage was used to infect *E. coli* strain P2392 to obtain plaques. 5x10⁴ plaque forming units were screened with phytase oligo PHY-1 using the following conditions: namely, 42°C overnight on filters in oligonucleotide hybridization solution (6X SSPE, 0.5% SDS, 3X Denhardt's, 100 µg/ml tRNA). Non-specific binding was removed by washing the filters twice for 30 minutes at room temperature with 2 X SSC, 0.1% SDS, and once for 5 minutes at 42°C in fresh solution. Overnight exposure on Kodak X-Omat AR film with intensifying screens revealed positively hybridizing plaques. Twelve strongly hybridizing plaques were picked for further characterization. Lambda phage with inserts that hybridized to the PHY-1 probes, and had an identical size to genomic DNA fragments on 0.8% agarose gel electrophoresis, were chosen for subcloning and eventual nucleotide sequencing (as described below, *Materials and Methods*).

Subcloning and sequencing of phytase genomic clones:

[0090] Bacteriophage DNA isolated essentially by the method of Yamamoto (1970) from each of the 12 candidates was digested with restriction endonucleases and probed with the PHY-1 oligonucleotide. A 1.8 kb BamHI/XbaI hybridizing fragment previously identified in genomic Southern's was isolated from clone number CH7 and subcloned into M13mp18 and M13mp19 [BRL]. Subclone CH7 that reacted positively with oligonucleotide probes for phytase (i.e., PHY-1) was sequenced.

[0091] The nucleotide sequence of this subclone revealed regions corresponding to the reported nucleotide sequence of other known phytase peptide sequences, thus confirming the probable identity of the clone as a phytase genomic DNA. Continued sequencing of an overlapping 2.6 kb SphI fragment revealed an open reading frame of 1409 bp that included 15 internal peptide sequences and N-terminal peptide sequence. Analysis of upstream sequence with the IntelliGenetics' PC/GENE program "SIGNAL" (based on the method of Standen, 1984), revealed a strong eukaryote Kozak consensus sequence followed by a methionine initiation codon. However, this ATG was out of frame with respect to the remainder of the sequence. A potential 102 bp intron delineated by consensus fungal donor, lariat and acceptor sequences (Rambosek and Leach, 1987) was identified between the potential initiation codon and the N-terminal peptide. Splicing of this putative intron restored the reading frame between the proposed ATG and the N-terminal peptide amino acid sequence. By incorporating this single putative intron in the 5' end of the phytase gene, the entire polypeptide was encoded by 470 amino acids. Sequence and translation of the phytase gene is shown in FIGURE 9.

FIGURE 9. Nucleotide sequence from the 2.6 kb SphI fragment including the phytase gene with deduced translation. The proposed intron donor (GTRNGT), lariat (RCTRAC) and acceptor (YAG) consensus sequences are overlined. The nucleotide sequence corresponding to peptide #420 (Table 3) is underlined. The nucleotide sequence was determined by the M13-dideoxy method (Sanger et al., 1977) of overlapping subclones with the use of the United States Biochemical Sequenase II kit.

[0092] The relative molecular mass of the translated phytase polypeptide was calculated at approximately 51,400

daltons. A codon usage analysis of phytase revealed a frequency of G+C at silent third position of sense codons (i.e., excluding the Trp and Met codons) of 68.3%. The entire structural gene and upstream sequence was subcloned as a 2.6 kb SphI fragment into *pUC-18* and designated *pFF-1* (FIGURE 10).

FIGURE 10. Circular transformation vectors of phytase (A) *pFF-1*: a 2.6 kb SphI fragment containing the phytase gene and its native promoter was subcloned from a positive lambda clone into *pUC-18*. An XbaI site was introduced at the -26 position of the phytase coding region in *pFF-1* by site directed mutagenesis to give *pSELFX*. (B) *pFF-2*: phytase under control of the *A. niger* β -tubulin promoter. A 2.0 kb XbaI fragment from *pSELFX* was ligated to a unique XbaI site in the fungal expression vector *pTL-113* to give *pFF-2*. (C) *pFF-3*: phytase under control of the *A. niger* GAPDH promoter. The 2.0 kb XbaI fragment from *pSELFX* was ligated to a unique XbaI site in *pPRE-81* to give *pFF-3*. (D) *pFF-4*: phytase under control of the *A. niger* GA promoter. The 2.0 kb XbaI fragment from *pSELFX* was ligated to a unique XbaI site in *pGA* to give *pFF-4*.

Isolation and characterization of the pH2.5 acid phosphatase gene:

[0093] The same lambda library was re-plated and screened with pH2.5 acid phosphatase oligonucleotide PHY-31 using conditions established with genomic hybridizations above. Twelve hybridizing plaques were picked for further characterization. Bacteriophage DNA isolated from each of the candidates was digested with restriction endonucleases and probed with either the PHY-31 oligo, or a second oligo mixture, PHY-34/35 that was derived from an independent pH2.5 acid phosphatase peptide (Table 5). One of the clones, AP99, contained a 2.1 kb SphI fragment previously identified in genomic Southern analysis, that hybridized strongly to both oligonucleotides. Strong hybridization to two oligonucleotides derived from independent peptide sequence strongly suggested that the clone contained pH2.5 acid phosphatase sequence. This fragment was subcloned into M13mp18 and M13mp19 for sequencing. The nucleotide sequence of this subclone revealed an ORF of 785 bp with a potential 5' initiation ATG (methionine), a fungal signal sequence, and a translated sequence compatible with the N-terminal peptide and other internal peptide sequences determined in Example 1, above (Table 4). Downstream of the ORF, termination codons were identified in all three reading frames until nucleotide 1151, after which an additional pH2.5 acid phosphatase peptide sequence was identified. These results necessitated the inclusion of an intron(s) in the 3' portion of the gene.

Identification of cDNA clones for pH2.5 acid phosphatase:

[0094] PolyA mRNA was purified (as described, below; *Materials and Methods*) and used for polymerase chain reaction (PCR) cloning of a portion of pH2.5 acid phosphatase cDNA. Briefly, first strand synthesis was performed with the oligonucleotide primer AP273 which was annealed to subspecies mRNA encoding pH2.5 acid phosphatase: namely, AP273 5' - CTACCCCTCTGCATCTAG - 3'. Oligonucleotide PCR primers UPPHOS and DOWNPHOS were synthesized with flanking EcoRI restriction sites: namely,

UPPHOS 5'-GAATTCCGAGTCCGAGGTCATGGGCGCG-3';

and,

DOWNPHOS 5'-GAATTCCCGGGACCTACCCCTCTGCAT-3'.

UPPHOS and DOWNPHOS were inversely oriented and separated by 978 bases in genomic clones. PCR amplification of the cDNA-mRNA complex with oligonucleotide primers UPPHOS and DOWNPHOS yielded a specific product of approximately 850 bps. The amplified product was isolated from agarose gels and digested with EcoRI. This fragment was then subcloned into *pUC-18* digested with EcoRI for double stranded sequencing.

[0095] PCR-amplified cDNA from the 3' portion of the gene was sequenced and revealed the presence of three short introns, each exhibiting consensus fungal donor, lariat and acceptor sequences. The exon sequence is derived by splicing the nucleotides 136-916, 971-1088, 1141-1245 and 1305-1740 (as shown in FIGURE 11). The resultant translated sequence codes for a protein of 479 aa. The entire sequence with translation is shown in FIGURE 11.

[0096] The deduced pH2.5 acid phosphatase polypeptide has a calculated molecular weight of approximately 52,700 daltons. A codon usage analysis of pH2.5 acid phosphatase revealed a very high frequency of G+C at silent third position of sense codons of 79.3%.

[0097] The 2.1 kb SphI fragment contained only 135 bp of upstream pH2.5 acid phosphatase sequence. Because this length of promoter sequence may not have been enough for efficient expression, a larger fragment was subcloned from the lambda clone. *pAP-3* contains a 5.1 kb Sall/PstI fragment from lambda clone AP99 and the nucleotide se-

quence is shown in FIGURE 11.

FIGURE 11. Nucleotide sequence from the 2.1 kb SphI fragment containing the pH2.5 acid phosphatase gene with deduced amino acid translation. The intron donor, lariat and acceptor sequences as determined by cDNA sequencing are overlined. The nucleotide sequence corresponding to peptides #816 and #1110 (Table 4) is underlined. The genomic nucleotide sequence was determined by the M13-dideoxy method (Sanger et al., 1977) with the use of the United States Biochemical Sequenase II kit.

Materials and Methods:

Enzymes:

[0098] Restriction enzymes were purchased from Bethesda Research Laboratories and New England Biolabs (Beverly, MA, USA). T4 DNA ligase, T4 DNA polymerase, T4 kinase, and the Klenow fragment from *E. coli* DNA polymerase I were purchased from BRL (Gaithersburg, MD, USA). Calf intestine phosphatase (CIP) was purchased from Boehringer Mannheim (Indianapolis, IN, USA). Spheroplasting enzyme, Novozyme, was purchased from Novo Biolabs (Bagsvaerd, Denmark). All enzymes were used in accordance with the manufacturer's recommendations. Enzyme assay substrates phytic acid and paranitrophenylphosphate (PNPP) were purchased from Sigma (St. Louis, MO, USA) and BMB, respectively.

Bacterial and fungal strains, plasmids and phage:

[0099] *A. niger* var. *awamori* strain ALKO243 (ATCC#38854) was used for isolation of the genes for phytase and pH2.5 acid phosphatase.

[0100] *E. coli* strains LE392 and P2392 and the phage Lambda Dash II, used in the construction of the *A. niger* gene bank, were obtained from Stratagene (La Jolla, CA, USA). *E. coli* strain JM109 was used as a host in transformations with constructions derived from the plasmids *pUC18* and *pUC19*. The phage M13mp18 and M13mp19 used in the dideoxynucleotide sequencing were obtained from Bethesda Research Laboratories (Gaithersburg, MD, USA).

[0101] Phleomycin resistant vector *pLO-3*, which contains the phleomycin resistance gene (a phleomycin binding protein gene from *Streptoalloteichus hindustanus*) coupled to a yeast cytochrome C1 terminator, was derived from the plasmid *pUT713* (CAYLA, Toulouse Cedex, France). It is expressed in fungus by the β -tubulin promoter of *A. niger*.

General growth media:

[0102] *E. coli* JM109 was grown in L-broth. Transformants were selected on L-plates supplemented with 1.5% agar and containing 125 μ g/ml ampicillin. Complete medium (CM) for growth of fungus in liquid is composed of: 50ml of 20X Clutterbuck's salts (120g NaNO₃, 10.4g KCl, 10.4g MgSO₄ 7H₂O, 30.4g KH₂PO₄), 2.0ml Vogel's Trace Elements (0.3M citric acid, 0.2M ZnSO₄, 25mM Fe(NH₄)₂(SO₄)₂·6H₂O, 10mM CuSO₄, 3mM MnSO₄·H₂O, 8mM boric acid, 2mM Na₂MoO₄·2H₂O), 5.0g tryptone, 5.0g yeast extract, 10g glucose, in one liter of distilled water. *A. niger* strains ALKO243 and ALKO2268 were grown on PD agar slants (2.4% Potato Dextrose Broth [Difco #0549]; 1.5% Agar [Difco #0140]) for seven to twelve days at 28°C.

Isolation of genomic DNA from *A. niger* var. *awamori* strain ALKO243:

[0103] One slant of ALKO243 grown on PDA for 5 days at 35°C was soaked with 5 ml of NP-40 H₂O (0.005% (v/v) Nonidet P40 detergent). Spores were scraped from slants and macerated in glass tubes with 3mm glass beads. 1 x 10⁸ spores were used to inoculate 500ml CM in a 2-liter flask. Cultures were grown at 35°C with shaking at 200 RPM for 48 hours. Mycelia were collected on mira-cloth, frozen in liquid nitrogen and lyophilized overnight. All of the frozen dried mycelia (approximately 2.0 grams) were ground with sea sand in a mortar chilled with liquid nitrogen. Ground mycelia were transferred to a 250ml centrifuge bottle and resuspended in 30ml 4% SDS-TE buffer (10mM Tris Base, 1mM EDTA, 4% SDS) and allowed to lyse at room temperature for 1 hour. Cell debris were removed by centrifugation at 4000xg for 5 minutes and the supernatant was removed to a 30ml centrifuge tube. The samples were extracted twice with an equal volume of Tris-saturated phenol:chloroform (1:1) each time removing the aqueous phase to a clean tube. DNA was precipitated by adding 10% (v/v) 5M NH₄OAc and 2.5 volumes of EtOH, and incubating overnight at -80°C. The preparation was thawed at room temperature until "syrupy" and was centrifuged at 12000xg for 30 minutes at 4°C. The supernatant was removed and the pellet dissolved in 19ml TE (10mM Tris Base, 1mM EDTA) with gentle pipeting, to which was added 19.0g CsCl. Two 11.5ml ultracentrifuge tubes were filled with DNA in TE + CsCl, 0.9ml of 10mg/ml ethidium bromide was added and the mixture was centrifuged at 45,000 RPM (20°C) for 22 hours in a Sorvall T865.1 rotor. Banded genomic DNA was visible under UV light and collected through an 18m gauge hypodermic

needle. Ethidium was removed by extraction with NaCl saturated isopropanol. CsCl was removed by dialysis against TE. DNA was precipitated with ethanol in 0.3M NaOAc.

Construction of genomic gene bank:

[0104] Genomic DNA from *A. niger* var. *awamori* strain ALKO243 was cut with Sau3A in order to produce fragments 10-23 kb in length. Cut DNA was ligated to purchased BamHI cut dephosphorylated Lambda Dash II vector arms. The ligation was packaged *in vitro* using Stratagene Gigapack Gold packaging extracts. Packaged phage was used to infect *E. coli* strain P2392 to obtain plaques.

Screening the gene bank with oligonucleotides:

[0105] Plaques were lifted onto Schleicher & Schuell NC (BA85) nitrocellulose membranes as recommended by the manufacturer. Using the amino acid sequences from phytase and pH2.5 acid phosphatase (Example 1, above), degenerate oligonucleotides were prepared for each protein. The oligonucleotide mixture for each protein was complementary to all possible codon combinations of the chosen region of the peptide. Oligonucleotides were synthesized using the Pharmacia Gene Assembler Plus and the sequences are shown in Table 5, above.

Isolation of Lambda DNA:

[0106] Single isolated hybridizing plaques were picked into 500ml SM (per liter: 5.8g NaCl, 2.0 g MgSO₄, 50 ml 1M Tris-HCl, pH7.5; 5 ml 2% (w/v) gelatin solution). 20ml chloroform was added and phage particles were eluted overnight at 4°C. 220ml of cell lysate was mixed with 200ml LE392 cells grown in the presence of Mg and maltose; 8ml NZCYM media (10.0g NZ-amine, 5.0g NaCl, 5.0g yeast extract, 2.0g MgSO₄·7H₂O, and 1.0g Casamino acids per liter; pH adjusted to pH7.5) was added; and, the culture was grown with shaking at 37°C until cells lysed (about 6 hours). 100ml chloroform was then added and incubation was continued for 15 minutes to ensure complete lysis. The sample was then centrifuged 5 min at 8000g and the supernatant was removed to fresh tube. RNase A and DNase I were added to 1 mg/ml each; the samples were incubated for 30 minutes at 37°C; and, an equal volume of 20% (wv) PEG 8000, 2M NaCl in SM was added to precipitate the phage. Samples were incubated for at least 1 hour on ice. The resultant phage were pelleted by centrifuging 10,000 x g for 20 minutes at 4°C; the supernatant was decanted; and, the phage pellet was resuspended in 0.5ml SM. DNA was purified further by addition of SDS, EDTA and Proteinase K, followed by extraction with phenol:chloroform and isopropanol precipitation. The sizes of the lambda DNA inserts were then examined on 0.8% agarose gels, the DNA was blotted to nitrocellulose and hybridized with the radiolabelled oligonucleotide PHY-1 or PHY-31 probes. DNA fragments that hybridize to the probes and had a size identical to genomic DNA fragments were chosen for subcloning.

Isolation of total RNA from *A. niger*:

[0107] Three 200ml cultures of *A. niger* var. *awamori* ALKO243 were grown in RNA broth media (2% corn starch [Sigma], 1% proteose peptone [Difco], 30g/l glucose, 5g/l NH₄NO₃, 0.5g/l MgSO₄·7H₂O, 0.5g/l KCl, 0.183g/l FeSO₄·7H₂O) for 48 hours at 30°C, 220 RPM. The mycelia were filtered through Whatman filter paper #1 and rinsed with 10ml of DEPC-treated H₂O (0.1% diethyl pyrocarbonate in sterile distilled water), then lyophilized overnight at -80°C. 1.5 grams (3g total) of dried mycelia was crushed under liquid nitrogen into a fine powder, then transferred to a centrifuge tube containing 10ml of Breaking Buffer (50mM Tris-HCl pH7.4, 150mM NaCl, 5mM EDTA pH8, 5% SDS) and 10ml of phenol:chloroform:isoamyl alcohol (P/CIA: 50:48:2). The mixture was allowed to thaw for thirty minutes at room temperature and was then centrifuged for 10 minutes at 10,000 RPM at 4°C. The aqueous layer was removed using a wide bore pipette into a clean centrifuge tube and re-extracted with P/CIA until the interface was clear. An equal volume of 6M LiCl was added to the final aqueous layer to precipitate the RNA and chilled overnight at -80°C. The mixture was then centrifuged for twenty minutes at 10,000 x g and 4°C, and the pellet was resuspended in 2.4ml of GTC solution (4M Guanidine thiocyanate, 25mM Sodium citrate pH7, 5% N-lauryl sarcosine, 100mM b-mercaptoethanol). An equal volume of isopropanol was added, the precipitate was chilled on dry ice for 40 minutes, and then centrifuged at 4°C for 30 minutes. The pellet was rinsed in 80% EtOH, re-spun, vacuum dried, and finally resuspended in 1ml of DEPC-H₂O. The concentration of RNA was determined spectrophotometrically at 260nm.

Isolation of mRNA:

[0108] Polyadenylated messenger RNA (polyA mRNA) was affinity purified from total RNA using oligo(dT)-cellulose columns according to the manufacturers instructions (Pharmacia Fine Chemicals, Piscatawy, N.J.). Briefly, 1.25mg of

total RNA was applied to two separate columns that were previously subjected to centrifugation and equilibration in High-salt buffer (Pharmacia). After three washes in Low-salt buffer (Pharmacia), the mRNA was eluted from the column at 65°C in prewarmed Elution Buffer (10mM Tris-HCl, pH7.4, containing 1mM EDTA); and precipitated by adding 0.1 volume of 10mg/ml glycogen and 2.5 volumes of ethanol. The mixture was chilled at -80°C for 2 hours, then centrifuged at 4°C for 30 minutes. The recovered mRNA was dissolved in Elution Buffer to a final concentration of 1.3mg/ml.

PCR isolation of pH2.5 acid phosphatase cDNA:

[0109] First strand synthesis was performed with the BMB cDNA kit according to manufacturers' recommendations with 1.0mg mRNA and oligonucleotide primer AP273, synthesized complementary to 3' pH2.5 phosphatase nucleotide sequence: namely, AP273 5'-CTACCCCTCTGCATCTAG-3'. Oligonucleotide PCR primers UPPHOS and DOWNPHOS were synthesized with flanking EcoRI restriction sites: namely,

UPPHOS 5' - GAATTCCGAGTCCGAGGTCATGGGCGCG - 3';

and,

DOWNPHOS 5' - GAATTCGCGGACCTACCCCTCTGCAT - 3',

as described above.

Subcloning and sequencing of phytase and pH2.5 acid phosphatase clones:

[0110] Hybridizing restriction fragments of lambda genomic clones were gel purified using the "Glassmilk Purification Kit" available commercially from GeneClean (Bio 101, La Jolla, CA). The restriction fragments were subcloned into M13mp-18 and M13mp-19 cut with the appropriate enzymes. The nucleotide sequence of clones reacting positively with oligonucleotide probes for phytase and pH2.5 acid phosphatase was determined by the M13-dideoxynucleotide sequencing method (Sanger et al., *Proc. Nat. Acad. Sci. USA* **74**:5463-5467, 1977) using the United States Biochemical Sequenase II kit. cDNA was sequenced in regions of suspected introns by alkaline denaturation double stranded sequencing. The phytase gene was completely contained within a 2.6kb SphI fragment that was subcloned into *pUC-18* to give *pFF-1*. The complete pH2.5 acid phosphatase gene was isolated as a 2.1bp SphI fragment that was subcloned into *pUC-18* to give *pAP-1*.

[0111] *pSELFX*, is a plasmid containing a phytase nucleotide sequence in which an XbaI site was introduced into the gene sequence by modifying the nucleotides at position -26 and -24 relative to the ATG start codon. The nucleotide changes were introduced by site-specific mutagenesis of the 2.6kb SphI fragment containing the phytase gene, using the Promega Altered Sites Mutagenesis Kit. The oligonucleotide used for directing mutagenesis is shown below: namely,

Oligo PHY-40: 5' -AGAAGAAATTTCTAGAACAGCAGCGATTGG - 3'
(XbaI)

[0112] *pAP-1Xba* is a plasmid containing a pH2.5 acid phosphatase nucleotide sequence in which an XbaI site was introduced into the gene sequence by modifying the nucleotides at positions -24 and -27 relative to the ATG start codon. The nucleotide changes were introduced by PCR-mutagenesis. The primers for PCR-mutagenesis are shown below: namely,

Oligo #271 5' - CGAGAGCACCTTCTCTAGATTTTGTCAAATGTACC - 3'
(XbaI)

and,

Oligo #272 5' - ACCCTCACCGAACTTGCGGGCCG - 3'.

[0113] *pAP-1* DNA was used as a template for PCR amplification. The resulting amplified fragment was cleaved with XbaI and NruI and the fragment was then ligated to XbaI/NruI cut *pAP-1* to give plasmid *pAP-1Xba*. The plasmid was sequenced to insure that no mutations were introduced into the region which had been PCR amplified. *pAP-2* was generated by ligating the BamHI/HindIII fragment treated with Klenow reagent from *pAP-1XBA* into SmaI cut *pUC-18*. Transformants containing the correctly oriented *pAP-2* were identified by analysis of the size of restriction endonuclease fragments.

[0114] The entire structural gene for pH2.5 acid phosphatase was removed from *pAP-2* as a 2.0kb XbaI fragment for construction of pH2.5 acid phosphatase transformation vectors that over-expressed the subject enzyme (Example 4, below). The complete pH2.5 acid phosphatase gene was also isolated as a 5.0kb PstI/SalI fragment that was sub-cloned into *pUC-18* to give *pAP-3*. *pAP-3* was used in the construction of pH2.5 acid phosphatase transformation vectors for over-expressing the subject enzyme (Example 4, below).

Northern blotting procedure:

[0115] Ten and twenty mg amounts of total RNA (isolated as above) were applied to wells of a 1.5% agarose-formaldehyde gel and separated by electrophoresis. RNA molecular weight markers (0.24kb-9.5kb "RNA Ladder," Bethesda Research Labs) were also applied to the gel for size comparisons. The gel was blotted onto nitrocellulose (Schleicher and Schuell; Keene, NH), baked for one hour at 80°C in a vacuum oven, prehybridized in 50% formamide, 5X SSC, 0.1% SDS, 5X Denhardt's and 100mg/ml calf thymus DNA at 37°C for 24 hours, then hybridized in the same solution with radiolabelled probe at 42°C for 24 hours. The filter was washed twice in 2X SSC/0.1% SDS at room temperature for fifteen minutes, then once in 0.1X SSC/0.1% SDS and autoradiographed using Kodak S-Omat AR film with intensifying screens.

EXAMPLE 3

Homology with other phosphatases

[0116] A comparison was made between the amino acid sequence of the pH2.5 acid phosphatase and phytase and other published acid phosphatase sequences. Homology (alignment score of 17.705) was determined using the PC/GENE matrix program PCOMPARE (based on the method of Needleman and Wunsch, 1970). Significant homology was found between the pH2.5 acid phosphatase and other acid phosphatase enzymes "PHO-3" (Bajwa, et al., 1984) and "PHO-5" (Arima, et al., 1983), both isolated previously by others from *Saccharomyces cerevisiae*. The area of highest homology, **R H G X R X P** (aa positions 81-87), was used to search the EMBL CDPROT17 protein database release 1991. The RHGXRX sequence was found in several other acid phosphatases (as shown in Table 6, below).

TABLE 6
Homology of phytase and pH2.5 Acid Phosphatase
with Other Acid Phosphatases^a

Organism Gene Name ^b		Amino Acid Sequence																	
<i>A. niger</i>	Phytase	A	Q	V	L	S	R	H	G	A	R	Y	P	T	E	S	K	G	K
<i>A. niger</i>	pH2.5 AP	V	I	M	V	K	R	H	G	E	R	Y	P	S	P	S	A	G	K
<i>E. coli</i>	APPA	V	V	I	V	S	R	H	G	V	R	A	P	T	K	A	T	Q	L
Yeast	PHO-1	V	H	T	L	Q	R	H	G	S	R	N	P	T	G	G	N	A	A
Yeast	PHO-5	L	Q	M	V	G	R	H	G	E	R	Y	P	T	V	S	L	A	K
Yeast	PHO-3	L	Q	M	L	A	R	H	G	E	R	Y	P	T	Y	S	K	G	A
Rat	LAP	V	T	L	L	Y	R	H	G	D	R	S	P	V	K	A	Y	P	K
Rat	PAP	V	T	L	V	F	R	H	G	D	R	G	P	I	E	T	F	P	N
Human	ACP2	V	T	L	L	Y	R	H	G	D	R	S	P	V	K	T	Y	P	K
Human	ACPP	V	T	L	V	F	R	H	G	D	R	S	P	I	D	T	F	P	T

a.) Homology with other acid phosphatases found using the target sequence RHGXRXRP with IntelliGenetics PC/GENE program QGSEARCH on the EMBL CDPROT17 protein database using default search parameters; **BOLD** residues are identical; b.) APPA = A pH2.5 acid phosphatase from *E. coli* ([Touati and Danchin, 1987]; PHO-1 = An acid phosphatase from *Scizosaccharamyces pombe* ([Elliot et al., 1986]); PHO-5 = repressible acid phosphatase from *Saccharomyces cerevisiae* ([Arima et al., 1983]); PHO-3 = non-repressible acid phosphatase from *S. cerevisiae* ([Bajwa et al., 1984]); LAP = rat lysosomal acid phosphatase ([Himeno et al., 1989]); PAP = a rat prostatic acid phosphatase ([Roiko et al., 1990]); ACP2 = a human lysosomal acid phosphatase ([Pohlmann et al., 1988]); ACPP = a human prostatic acid phosphatase ([Tailor et al., 1990]).

[0117] The RHGXRXRP region is conserved between organisms as diverse as *E. coli* and humans. This conservation suggests functional significance and thus may reflect an active site region of these phosphatases.

EXAMPLE 4

Over-expression of recombinant phytase and pH2.5 acid phosphatase in *Aspergillus niger*

[0118] [General materials and methods are described in the section entitled "Materials and Methods," which follows this example.]

Construction of expression vectors phytase:

[0119] A number of vectors were designed for the reintroduction of phytase and pH2.5 acid phosphatase using both native and alternative fungal promoters. One set of vectors carrying the phytase gene, *pFF1* - *pFF4* (FIGURE 10, above), contained no fungal selectable marker and were introduced by co-transformation with the plasmid *pL03* (see *Materials and Methods*, below).

[0120] Recombinant strains resulting from vectors *pFF1*-2 contained foreign DNA sequences derived from the *E. coli* selectable marker. Therefore, a second set of vectors termed *pFF6*-*pFF11* was designed to remove these sequences. The latter vectors contained a fungal selectable marker and restriction sites allowing linear fragments to be isolated free of *E. coli* sequences (FIGURE 12).

FIGURE 12. Linear transformation vectors of phytase. (A) *pFF-6A*: phytase under control of its native promoter. The 2.6 kb *SphI* fragment from *pFF-1* was cloned into the *SphI* site of *pL03* to give *pFF-6*, was isolated as a linear 4.9 kb *HindIII* fragment. (B) *pFF-8*: phytase under control of the GAPDH promoter. The phleomycin resistance cassette from *pL03* was isolated as a *HindIII* (filled)/*PstI* fragment and subcloned into *BglII* (filled)/*PstI* cut *pPRE-81* to give *pPRE-82*. The *pSELF* *XbaI* fragment containing phytase was added to *pPRE-82* partially cut with *XbaI* to give *pFF-8*, that was isolated as a 6.7kb *PstI* fragment. (C) *pFF-9*: phytase under control of the GA promoter. The phleomycin resistance cassette from *pL03* was isolated as a *KpnI* (filled)/*HindIII* fragment and ligated to *pFF-4* partially cut with *KpnI*, blunted, then cut with *HindIII*. *pFF-9* was isolated as a 7.3 *HindIII*/*KpnI* linear fragment. (D) *pFF-11*: phytase under control of the

GA promoter and signal sequence. Oligos #260 and #261 (see *Materials and Methods*, below) were annealed and ligated to XbaI/XhoI digested *pFF-1*, the GA promoter added as a KpnI/XbaI fragment, and the phleomycin resistance marker (Phleo^r) cassette was added as a HindIII fragment to give *pFF-11*. Linear DNA was isolated as a 7.35 Kbp fragment.

Construction of expression vectors; pH2.5 acid phosphatase with and without phytase:

[0121] Transformation vectors for pH2.5 acid phosphatase consisted of linear vectors, termed *pPH01* - *pPH04A*, that were constructed for introduction of the pH2.5 acid phosphatase gene (FIGURE 13). Two vectors, *pFIN-1A* and *pFIN-1B*, were also engineered to contain both the phytase and pH2.5 acid phosphatase genes expressed from the glucoamylase promoter in a single plasmid (FIGURE 14).

FIGURE 13. Vectors from which linear fragments can be isolated for the reintroduction of pH2.5 acid phosphatase. (A) *pPHO-1*: pH2.5 acid phosphatase under control of its native promoter. The phleo^r cassette was isolated as a HindIII fragment, blunt-ended, and ligated to *pAP-3* (the pH2.5 acid phosphatase gene in a 5.0kb PstI/Sal fragment in *pUC-18* (5)), cut with EcoRI and also blunt-ended. Linear DNA was isolated as a 6.3kb PstI fragment. (B) *pPHO-2*: pH2.5 acid phosphatase under control of the β -tubulin promoter. The pH2.5 acid phosphatase gene contained in a 2.0kb XbaI fragment from *pAP-1Xba* (*Materials and Methods*, below) was subcloned into *pTL-113*, which was then partially cut with SphI, filled, and ligated to a fragment containing the phleomycin-resistance marker cassette, yielding *pPHO-2*. (C) *pPHO-3*: pH2.5 acid phosphatase under control of the GAPDH promoter. *pPHO-2* was partially digested with XbaI to give a 4.3 kb XbaI fragment containing pH2.5 acid phosphatase and the phleo^r cassette, which was then ligated into the XbaI site of *pPRE-81*. (D) *pPHO-4A*: pH2.5 acid phosphatase under control of the GA promoter. The 2.0kb XbaI fragment from *pAP-1Xba* was subcloned into *pGA*, and a 5.5kb KpnI fragment containing the GA promoter and pH2.5 acid phosphatase was isolated and filled in and then ligated into *pLO3*.

FIGURE 14. A dual-enzyme-transformation vector was constructed for over-expression of both phytase and pH2.5 acid phosphatase by a single plasmid. (A) *pFIN-1A* and (B) *pFIN-1B*: combination plasmids with both phytase and pH2.5 acid phosphatase under control of separate copies of the GA promoter. An 8.1kb HindIII fragment was isolated from *pPHO-4A* and ligated into HindIII-digested *pFF-4*.

Transformation vector constructs: general considerations:

[0122] As is evident from the information in the discussion above of FIGURES 12-15, several different fungal promoter constructions were evaluated: namely, the β -tubulin promoter, the glyceraldehyde 3-phosphate dehydrogenase promoter (GAPDH), and the glucoamylase promoter (GA). The latter two fungal promoters were isolated from *A. niger* ATCC1015 (Tailor, P. et al. 1990). These respective promoters were tested to determine their ability to drive expression of phytase and pH2.5 acid phosphatase.

[0123] To facilitate fusion of alternative promoters into the constructs, an XbaI restriction site was introduced by site-directed mutagenesis at the -26 position of the phytase gene. Similarly, an XbaI site was engineered at position -28 of the pH2.5 acid phosphatase gene by PCR mutagenesis (see *Materials and Methods*, below). The engineered XbaI sites were subsequently used for fusions to alternative fungal promoters previously engineered to contain such XbaI sites in the 5' regulatory regions of the gene.

[0124] The results obtained in cells transformed with phytase vector constructs are presented first, below, followed by the results obtained with pH2.5 acid phosphatase.

Transformation of *A. niger* strains ALKO243 and ALKO2268:

[0125] *A. niger* var. *awamori* strain ALKO243 and *A. niger* strain ALKO2268, a mutant strain exhibiting higher production of phytase derived from ALKO243 by UV-mutagenesis, were used as hosts for engineered phytase and pH2.5 acid phosphatase. *A. niger* strains ALKO243 and ALKO2268 were transformed by a modification of the procedure of Tilburn et al. (1983) using phleomycin resistance as a drug-selectable genetic marker. Because the cloned phytase and pH2.5 acid phosphatase genes were maintained on plasmids without a selectable marker, spheroplasts were co-transformed with a phleomycin resistant vector *pLO-3* that contains the phleomycin binding protein gene from *Streptococcus hindustanus* coupled to a yeast cytochrome C1 terminator. *pLO-3* was derived from the plasmid *pUT713* [CAYLA, Toulouse Cedex, France] and is expressed in fungus by the β -tubulin promoter of *A. niger*. Transformants were selected with an equal volume overlay containing 50 μ g/ml phleomycin for strain ALKO243 and 195-200 μ g/ml for strain ALKO2268. (Strains ALKO243 and ALKO2268 were both sensitive to spheroplast lysis, and required different osmotic buffering conditions; see *Materials and Methods*, below.) Thirty to forty-five sporulating colonies were obtained per μ g of DNA on selection plates. All phleomycin resistant colonies had integrated the phleomycin resistance marker (Southern analysis, data not shown).

Screening of transformants for over-expression of phytase:

[0126] Transformants producing higher phytase activity than parental *A. niger* strain ALKO243 were identified in a phytase plate screening assay that colorimetrically measures the accumulation of inorganic phosphate by the reduction of a phosphomolybdate complex (*Materials and Methods*, below). Transformants with high activity in the plate assay were subsequently tested for phytase production by assaying the enzyme activity released into the broth of a fermentation culture (as described, below).

FIGURE 15 shows a plate assay for detecting increased phytase activity. Conidia from *A. niger* phytase transformants GAD-10 through GAD-120 was spotted onto plate assay media, incubated for two days at 30°C, and overlaid with Reagent C for color detection (*Materials and Methods*, below). Positives GAD 130 (a4), GAD 112 (e3) and GAD 118 when grown in shaker flask cultures, exhibited titers of 108,000PU/ml, 35,600PU/ml, and 36,700PU/ml, respectively (i.e., increases of about up to about 1260-fold higher than ALKO243). Phytase activity for untransformed ALKO2268 (b5) was 4,000PU/ml.

Analysis of phytase over-producing transformants:

[0127] The amount of phytase produced by transformants was analyzed by growing the cells in shaker flask cultures and titrating the amount of enzyme in the broth, as follows: briefly, cultures were established in shaker flasks under optimal conditions (*Materials and Methods*, below) and incubated for 5 days, at which time the cells were removed by centrifugation and the supernatant broth from the culture was titrated to determine the amount of phytase activity. (Both the plate assay (above) and the broth titer assay measured the amount of inorganic phosphate released by enzymatic hydrolysis of sodium phytate substrate by colorimetrically quantifying the reduction of a phosphomolybdate complex.)

[0128] Using the native phytase promoter (from plasmid; *pFF-1* see, Example 2, *Materials and Methods*, above), of the 58 transformants generated, four exhibited increased production of phytase that was 6.5- to 7.0-fold greater than that produced by non-transformed over-producer strain ALKO2268 ("Untransformed", Table 7, below) or by ALKO2268 control cells that were transformed with a control plasmid *pLO-3* which contained no phytase sequences.

TABLE 7

Over-expression of phytase in transformants of production strain ALKO2268 ^a				
Strain	Plasmid	Promoter	Activity ^b	Increase ^c
ALKO2268 Untransformed	---	Native	4,000	---
GAD17	<i>pFF-1</i>	Native	26,000	6.5
GAD33	<i>pFF-1</i>	Native	17,500	4.4
GAD40	<i>pFF-1</i>	Native	10,500	2.6
GAD48	<i>pFF-1</i>	Native	28,000	7.0

a.) Recombinant ALKO2268 over-producers of either phytase in shake flask cultures, i.e., fermentation in a soy flour cultivation media, after 5 days of shaking at 28°C. Cellular and media components were removed by centrifugation. Enzyme activity was determined by adding dilutions of the culture broth supernatant to 1% sodium phytate [Sigma], in 0.2M citrate buffer, pH5.0. After fifteen minutes at 37°C, each reaction was terminated by adding 15% TCA (trichloroacetic acid, [Merck]). Released orthophosphate was measured by adding an equal volume of Reagent C (3:1:1 ratio of 1M sulfuric acid [Mallinckrodt], 2.5% ammonium molybdate (Sigma), 10% ascorbic acid [Sigma]) to a 1:10 dilution of the hydrolysis reaction and incubating at 50°C for twenty minutes. Absorbance of the reaction mixture was measured against a reagent blank and known phosphate standards (1:100 - 1:400 dilutions of 9.0mM KH₂PO₄) at 820nm;

b.) Activity is expressed as phytase units per ml (i.e., PU/ml);

c.) Increase is expressed as the fold increase in activity (i.e., in PU/ml) over the activity (i.e., in PU/ml) with ALKO2268 (i.e., activity of transformant/activity of ALKO2268).

[0129] Further studies involved use of heterologous promoters driving phytase expression 1467 phytase transformants were examined using the plate assay (157 were derived from strain ALKO243, and 1310 were from strain ALKO2268). Of these, 302 were identified in the plate assay as being potential over-producers of phytase. Of the phytase-over-producing transformants, 25 (i.e., 1.7% of the 1467 evaluated) had enzyme activity in excess of 700-fold greater PU/ml than that produced by ALKO243. (The results obtained with 18 of the 25 transformants are shown in Table 8.) The transformant exhibiting the highest phytase production (i.e., GAI-6; 181,000PU; about 2,100-fold greater PU/ml than ALKO243) was the result of transformation with the *pFF-9* construct containing a glucoamylase (GA) fungal promoter. The latter transformant showed significantly increased levels of phytase production compared to ALKO2268 (an over-producer) or to ALKO243 (a wild-type strain) (see Table 8, below).

TABLE 8

<i>A. niger</i> phytase transformants which produced phytase enzyme over 700-fold greater than ALKO243 in shake flask cultures*					
Transformant	Strain	Plasmid ^a	Promoter	Activity ^b	increase ^c
GAI-6	A.2268	<i>pFF-4</i>	GA	181,000	2129
GAL-142	A.2268	<i>pFF-3</i>	GAPDH	174,000	2047
GAN-1	A.2268	<i>pFF-8</i>	GAPDH	170,000	2000
GAG-12	ALKO243	<i>pFF-3</i>	GAPDH	166,000	1953
GAO-248	A.2268	<i>pFF-9</i>	GA	164,000	1929
GAI-12	A.2268	<i>pFF-4</i>	GA	145,000	1706
GAK4-46	A.2268	<i>pFF-4</i>	GA	132,000	1553
GAI-2	A.2268	<i>pFF-4</i>	GA	131,000	1541
GAK4-52	A.2268	<i>pFF-4</i>	GA	131,000	1541
GAM-111	A.2268	<i>pFF-6A</i>	NATIVE	129,000	1518
GAK4-47	A.2268	<i>pFF-4</i>	GA	121,000	1424
GAM-225	A.2268	<i>pFF-9</i>	GA	112,000	1318
GAD-103	A.2268	<i>pFF-3</i>	GAPDH	108,000	1271
GAD-23	A.2268	<i>pFF-3</i>	GAPDH	108,000	1271
CAM-199	A.2268	<i>pFF-9</i>	GA	107,600	1266
GAE-32	A.2268	<i>pFF-6A</i>	NATIVE	72,600	854
GAL-65	A.2268	<i>pFF-9</i>	GA	72,200	849
GAE-3	A.2268	<i>pFF-6A</i>	NATIVE	67,500	794
Control	ALKO2268	None	Native	3,800	45
Control	ALKO243	None	Native	85	1

a.) Plasmids *pFF-9*, *pFF-8* and *pFF-6A* were used in linear form with all *E. coli* sequences excised Transformant designation; GA = glucoamylase promoter; GAPDH = glyceraldehyde 3-phosphate dehydrogenase promoter; NATIVE = native phytase promoter;

b.) = Activity from shake flask fermentation culture; Enzyme activities (i.e., in PU/ml) are averages of two independent fermentations;

c.) Increase is expressed as the fold increase in activity in shake flask culture over the activity with ALKO243 in shake flask culture (i.e., activity in PU/ml of transformant per activity in PU/ml of ALKO243).

Molecular characterization: phytase transformants:

[0130] Southern and Northern analyses were conducted with DNA and mRNA (respectively), isolated from phytase transformants GAE 32, GAK 4-47, GAL 65, GAM 225 and GAO 248 (Table 8) that over-produced phytase in shake flask fermentation cultures. Genomic DNA was isolated from the respective transformant strains, digested with restriction enzymes, and probed with a radiolabelled cloned phytase DNA restriction fragment (i.e., isolated from *pFF1*, above), to determine the gene copy number and the method of integration in the transformants. An increase in gene copy number was determined by comparing the density of the blot at 1.3kb (i.e., the transformation vector construct DNA) against the density of the endogenous chromosomal copy of the phytase gene (i.e., at 2.4kb) and against control ALKO2268 genomic DNA. Quantification was achieved by scanning densitometry (FIGURE 16A). Three to five additional phytase vector gene copies, arranged in multiple different patterns of integration (data not shown), were detected in transformants GAE 32, GAK 4-47, GAL-65, GAM-225 and GAO-248.

[0131] Expression of phytase mRNA levels by Northern blotting demonstrated that message levels of transformants were consistently increased by seven- to thirteen-fold over non-transformed controls (FIGURE 16B). However, actual levels may be higher because it proved difficult to isolate mRNA from cells grown in production media, and it was therefore necessary to make these comparisons under sub-optimal conditions for cell growth and phytase expression.

FIGURE 16A. Southern blot analysis of phytase genomic copy number in over-producing phytase transformants: genomic DNA was digested with BamHI, blotted, electrophoresed, and the filter was probed with BamHI fragments of the 5' region of *pFF-1*. Gene copy number was determined by comparing blot density of gene copies (at 1.3kb) in transformants with the blot density of the endogenous phytase gene (at 2.4kb) by scanning densitometry. Lanes 1, 4, 7: Untransformed control ALKO2268. Lanes 2, 5, 8, 9, 10: over-producing phytase transformants GAE-32, GAK-47, GAL-65, GAM-225, GAO-248, respectively.

FIGURE 16B. Northern blot analysis messenger RNA levels in phytase over-producing transformants: 20 µg of

each total RNA sample was electrophoresed in a formaldehyde gel and transferred to nitrocellulose. Each transformant was probed with the restriction fragments of the phytase gene (i.e., BamHI fragments from *pFF-1*). Increased phytase message level was measured by scanning densitometry of the mRNA, RNA from ALKO2268 (Lane 1) was used as baseline control. Lanes 2-6: GAE-32, GAK-47, GAL-65, GAM-225, GAO-248, respectively.

[0132] The results presented in Table 9, below, summarize the quantitative results obtained by scanning the density of the Southern and Northern blots in FIGURE 16A and FIGURE 16B.

TABLE 9

Comparison of phytase gene copy number, mRNA levels and enzyme production by phytase over-producers					
Strain	Plasmid ^a	Copy No.	mRNA ^b	Activity ^c	Increase ^d
ALKO243	None	1.0	--	85	1
ALKO2268	None	1.0	1.0	3.800	45
GAO-248	<i>pFF9</i> (GA, lin)	6.0	9.2	164.000	1,929
GAK-47	<i>pFF4</i> (GA, clr)	3.0	13.2	121,400	1,428
GAM-225	<i>pFF9</i> (GA, lin)	3.0	7.6	112.000	1,318
GAE-32	<i>pFF6</i> (nat, clr)	6.0	11.3	72,600	854
GAL-65	<i>pFF9</i> (GA, lin)	3.0	11.8	72.200	849

a.) nat = native promoter; clr = circular vector; lin = linear DNA fragment; GA = glucoamylase promoter;

b.) mRNA = messenger RNA amplification levels (as fold-increase over levels in ALKO243);

c.) Activity = phytase production in fermentation culture represented in phytase units per ml (i.e., PU/ml), conditions described in Table 7, above;

d.) = Increase is expressed as the fold increase in activity in shake flask culture over the activity with ALKO243 in shake flask culture (i.e., activity in PU/ml of transformant per activity in PU/ml of ALKO243).

Analysis of over-producing pH2.5 acid phosphatase transformants:

[0133] It was not possible to develop a plate screening assay specific for pH2.5 acid phosphatase; therefore, transformants were analyzed by evaluating enzyme production in the culture broth after 5 days of fermentation in shake flasks (as described above, Table 7, and in the *Materials and Methods*, below). The conditions for measuring production of pH2.5 acid phosphatase in the fermentation shake-flask assay are described below (Table 10) using paranitrophenylphosphate as the substrate (also described below in the *Materials and Methods*). As in Table 7, above, the levels of enzyme in the culture broth supernatant were titrated and the levels of enzyme were compared with the levels produced over the same 5-day period of incubation by a control culture of the ALKO243 strain of *A. niger*.

[0134] Of the 55 transformants generated with the plasmid *pAP-3* containing the native promoter driving expression of pH2.5 acid phosphatase (Example 2, *Materials and Methods*, above), four transformants showed increases in production of pH2.5 acid phosphatase that was 25- to 57-fold greater (Table 10) than the levels produced by parental strain ALKO243. The highest producing transformant (GAQ56) exhibited nearly a 58-fold increase over untransformed ALKO2268 (Table 10), and up to a 126-fold increase is illustrated in Table 11, below.

TABLE 10

Over-expression of pH2.5 acid phosphatase in transformants of production strain ALKO2268 ^a				
Strain	Plasmid	Promoter	Activity ^b	Increase ^c
Untransformed: A.2268	None	Native	2.300	—
Transformed: GAQ56	<i>pAP-3</i>	Native	133,000	57.5
GAO13	<i>pAP-3</i>	Native	102.500	44.5
GAQ66	<i>pAP-3</i>	Nauve	77,800	33.8
GAQ62	<i>pAP-3</i>	Native	57,600	25.0

a.) Conditions, as described in Table 7, above;

b.) Activity in acid phosphatase units per ml (i.e., HFU/ml);

c.) Increase is expressed as the fold increase in activity in shake flask culture over the activity with ALKO2268 in shake flask culture (i.e., activity in HFU/ml of transformant per activity in HFU/ml of ALKO2268).

[0135] Further studies evaluated heterologous promoters driving pH2.5 acid phosphatase expression. 1181 trans-

formants over-produced pH2.5 acid phosphatase (410 resulted from strain ALKO243 and 771 were from strain ALKO2268). 32 transformants (2.7%) were identified that produced pH2.5 acid phosphatase in excess of 30 HFU/ml. The highest producing transformant (i.e., GAO-69) averaged 126 HFU/ml. This expression resulted from transformation with a plasmid construct having a fungal glucoamylase (GA) promoter (Table 11).

TABLE 11

<i>A. niger</i> pH2.5 acid phosphatase transformants over-producing enzyme at levels over 40-fold higher than ALKO243 in shake flask fermentation cultures ^a					
Transformant	Strain	Plasmid	Promoter	Activity ^b	Increase ^c
GAO-69	A.2263	<i>pPREPHO-4</i>	GA	693,000	126
GAW-131	A.2268	<i>pPHO-4A</i>	GA	583,000	106
GBL-128	ALKO243	<i>pPHO-3</i>	GAPDH	566,500*	103*
GBL-97	ALKO243	<i>pPHO-3</i>	GAPDH	533,500*	97*
GAO-61	A.2268	<i>pPREPHO-4</i>	GA	451,000	82
GAW-89	A.2268	<i>pPHO-3</i>	GAPDH	412,500	75
GAW-130	A.2268	<i>pPHO-4A</i>	GA	385,000	70
GAW-121	A.2268	<i>pPHO-4A</i>	GA	379,500	69
GBL-87	ALKO243	<i>pPHO-3</i>	GAPDH	346,500*	63*
GBL-119	ALKO243	<i>pPHO-3</i>	GAPDH	335,500 *	61*
GAO-84	A.2268	<i>pPREPHO-4</i>	GA	330,000	60
GAW-54	A.2268	<i>pPHO-3</i>	GAPDH	280,500	51
GBL-129	ALKO243	<i>pPHO-3</i>	GAPDH	264,000*	48*
GAW-141	A.2268	<i>pPHO-4A</i>	GA	258,500	47
GBL-103	ALKO243	<i>pPHO-3</i>	GAPDH	242,000*	44 *
GAW-112	A.2268	<i>pPHO-4A</i>	GA	236,500	43
GBL-92	ALKO243	<i>pPHO-3</i>	GAPDH	236,500*	43*
GAW-114	A.2268	<i>pPHO-4A</i>	GA	231,000	42
GAT-143	ALKO243	<i>pPHO-4A</i>	GA	231,000	42
Control	ALKO2268	None	native	2,300	0.5
Control	ALKO243	None	native	5.500	1

a.) Transformed ALKO2268 and ALKO243 over-producers of pH2.5 acid phosphatase in shake flask fermentation cultures (conducted as described in Table 7, above). Enzyme activity was determined by adding dilutions of the culture broth supernatant to 30mM p-nitrophenyl phosphate (Boehringer Mannheim) in 0.2M glycine-HCL, pH2.5 buffer. The reaction was terminated and orthophosphate assayed as described in the footnote to Table 7. Enzyme activities are averages of activities from two independent fermentations except (*), which were from single fermentations. Plasmids *pPHO-3* and *pPHO-4A* were used in linear form with *E. coli* sequences excised. Transformant designation: GA = glucoamylase promoter; GAPDH = glyceraldehyde 3-phosphate dehydrogenase promoter;

b.) Activity in acid phosphatase units per ml (i.e., HFU/ml);

c.) Increase is expressed as the fold increase in activity in shake flask culture over the activity with ALKO243 in shake flask culture (i.e., activity in HFU/ml of transformant per activity in HFU/ml of ALKO243).

Molecular characterization of pH2.5 acid phosphatase transformants:

[0136] Copy number and message levels were determined using Southern and Northern blot analysis and scanning densitometry (as described above) for five of the highest pH2.5 acid phosphatase over-producers identified in the shake flask fermentation cultures (Table 11, above). DNA from pH2.5 acid phosphatase transformants GAT-143, GAW-54, GAW-89, GAW-121 and GAW-130 was digested with restriction enzymes and hybridized to a radiolabelled pH2.5 acid phosphatase-specific probe. Overall, the pH2.5 acid phosphatase transformants had higher gene copy numbers (Table 12, below) than observed above with the phytase transformants (Table 9, above). In contrast, message levels for pH2.5 acid phosphatase transformants (Table 12) were not as high as observed in phytase transformants (Table 9). However, as with phytase transformants above, the pH2.5 acid phosphatase message levels were not measured under optimal fermentation conditions because of the difficulty encountered in isolating RNA from cells under production culture conditions. Therefore, expression under production conditions may be higher than that measured here.

TABLE 12

Comparison of pH2.5 Acid Phosphatase Gene Copy Number with mRNA levels and Enzyme Production by Recombinant Transformed Strains ^a					
Strain	Plasmid	pH2.5 Acid Phosphatase			
		Copy No.	mRNA	Activity ^b	Increase ^c
A.2268	None	0	1.0	2,300	0.4
GAT-143	<i>pPHO4A</i> (GA, lin)	10.0	5.6	231,000	42.0
GAW-54	<i>pPHO3</i> (GAP, lin)	7.0	5.7	280,500	51.0
GAW-89	<i>pPHO3</i> (GAP, lin)	12.0	3.0	412,500	75.0
GAW-121	<i>pPHO4A</i> (GA, lin)	11.0	3.0	379,500	69.0
GAW-130	<i>pPHO4A</i> (GA, lin)	7.0	7.2	385,000	70.0

a.) GA = glucoamylase promoter; GAP = glyceraldehyde 3-phosphate dehydrogenase promoter; lin = linear DNA fragment; mRNA = messenger RNA amplification levels (as fold-increase over levels in ALKO243);

b.) = pH2.5 acid phosphatase activity represented in acid phosphatase units per ml (HFU/ml); comparisons made with cells grown in shake flask cultures; Activity = enzyme activity measured as described in Table 10, above;

c.) Increase is expressed as the fold increase in activity in shake flask culture over the activity with ALKO243 in shake flask culture (i.e., activity in HFU/ml of transformant per activity in HFU/ml of ALKO243).

Materials and Methods:

[0137] Plasmid constructions were designed so that vector backbone sequences could be easily removed prior to transformation of the respective genes into fungi. Linear DNA was isolated from agarose and purified by GeneClean to remove any possible contaminating specificities. Constructs A-D and Constructs E-H expressing the phytase and pH2.5 phosphatase genes, respectively, under different regulatory control were prepared as described below.

Phytase and pH2.5 acid phosphatase expression plasmids:

Construct A: *pFF-6* phytase under control of its native promoter.

[0138] The 2.6kb SphI fragment containing the phytase gene was cloned into the SphI site of *pLO-3*. Two orientations were generated, termed *pFF-6A* and *pFF-6B*. Linear DNA was isolated from either vector as a 4.9kb HindIII fragment.

Construct B: *pFF-8* Phytase under control of the *A. niger* GAPDH promoter.

[0139] Fungal expression vector *pPRE8-1* was cut with BglII and blunted with DNA polymerase I Klenow fragment; the plasmid was then cut with PstI to completion. The phleomycin resistance (Phleo^r) marker expression cassette was removed from *pLO-3* (as a HindIII (filled)/PstI fragment), and ligated to the cut *pPRE8-1* to give *pPRE8-2*. Transformants were identified by restriction analysis. The 2.0kb XbaI fragment containing the phytase gene (Example 2, *Materials and Methods*) was ligated into a unique XbaI site downstream from the GAPDH promoter in plasmid *pPRE8-2*. Correctly oriented transformants containing *pFF-8* were identified by restriction analysis. Linear DNA was isolated from *pFF-8* as a 6.7kb PstI fragment.

Construct C: *pFF-9* Phytase under control of the *A. niger* GA promoter.

[0140] The 2.0kb XbaI fragment containing the phytase gene (Example 2, *Materials and Methods*, above) was ligated to the unique XbaI site of fungal expression vector *pGA* to give *pFF-4*. Transformants containing correctly oriented plasmid were identified by restriction analysis. *pFF-4* was cut partially with KpnI to produce singularly cut linear plasmid. The ends were blunted with T4 DNA Polymerase. The plasmid was then cut to completion with HindIII. *pLO-3* was first cut with KpnI and blunted with T4 DNA Polymerase, and then cut with HindIII. The 2.1kb fragment containing the phleo^r expression cassette was purified from agarose with GeneClean and ligated to cut *pFF-4*. Transformants containing

pFF-9 were identified by restriction analysis. Linear DNA was isolated from *pFF-9* as an 7.3kb HindIII/KpnI fragment.

Construct D: *pFF-11* Phytase under control of the *A. niger* GA promoter with secretion via the GA signal sequence.

[0141] Oligonucleotides 260 and 261 were synthesized coding for an XbaI site upstream from the translation initiation region and signal sequence for glucoamylase, which was located, in turn, upstream from nucleotide sequence encoding the N-terminus of phytase (SEQ. ID. NO. 1). (The construct also contained a native XhoI site immediately downstream from this region.) The two synthetic oligonucleotides used in this construct had the following nucleotide sequence: namely,

Oligo260: 5'-CTAGACACCTCAGCAATGTCGTTCCGATCTCTACTCGCCCTGA
GCGGCCTCGTCTGCACAGGGTTGGCACTGGCAGTCCCCGCC-3' (84 mer); and,

Oligo261: 5'-TCGAGGCGGGGACTGCCAGTGCCAACCCTGTGCAGACGAGGC
CGCTCAGGGCGAGTAGAGATCGGAACGACATTGCTGAGGTGT-3' (84 mer).

[0142] Oligonucleotides 260 and 261 were annealed and then ligated to XbaI/XhoI cut *pFF-1* to give *pFF-1GA*. The glucoamylase promoter from pGA was ligated into *pFF-1GA* (as a KpnI/XbaI fragment) to give *pPREFF-11*. Finally, a phleo^r cassette was added as a HindIII fragment into a unique HindIII site of the construction. Transformants containing correctly oriented plasmid were identified by restriction analysis. Linear DNA was isolated from *pFF-11* as a 7.35 KpnI fragment.

Construct E: *pPHO-1* pH2.5 Acid Phosphatase (AP) under control of its native promoter.

[0143] *pAP-3* was cut with EcoRI and blunt-ended with Klenow. The phleo^r cassette was removed from *pLO-3* as a HindIII fragment, blunt-ended with Klenow, and ligated into the cut *pAP-3* plasmid. Transformants containing correctly oriented *pPHO-1* were identified by restriction analysis. Linear DNA was isolated from *pPHO-1* as a 6.3kb PstI fragment.

Construct F: *pPHO-2*, pH2.5 Acid Phosphatase gene under control of the *A. niger* b-tubulin promoter.

[0144] Fungal expression vector *pTL113* was cut with XbaI and dephosphorylated with calf intestinal phosphatase. A 2.0kb XbaI fragment containing the pH2.5 phosphatase gene (Example 2, *Materials and Methods*, above) was ligated into cut *pTL113* to give *pPREPHO-2*. Transformants containing correctly oriented plasmids were identified by restriction analysis. *pPREPHO-2* was partially cut with SphI to give singularly cut linear plasmid. The ends of cut *pPREPHO-2* were blunted with T4 DNA Polymerase. A 2.7kb blunt-ended HindIII fragment containing the phleo^r expression cassette from *pLO-3* was ligated into the cut *pPREPHO-2* vector. Transformants containing correctly oriented *pPHO-2* plasmid were identified by restriction analysis. Linear DNA was isolated as a 6.0kb PstI fragment

Construct G: *pPHO-3*, pH2.5 acid phosphatase gene under control of the *A. niger* GAPDH promoter.

[0145] A 4.3kb XbaI fragment containing the pH2.5 phosphatase gene (Example 2, above) and the phleo^r expression cassette was removed from *pPho-2* by partial digestion. The fragment was purified from agarose with GeneClean and ligated into a unique XbaI site in fungal expression vector *pPRE8-1*. Transformants containing correctly oriented *pPHO-3* plasmid were identified by restriction analysis. Linear DNA was isolated as a 7.0kb PstI fragment.

Construct H: *pPHO-4*, pH2.5 Acid Phosphatase gene under control of the *A. niger* GA promoter.

[0146] The 2.0kb XbaI fragment containing the pH2.5 acid phosphatase gene (Example 2, above) was ligated into XbaI cut fungal expression vector *pGA* to give *pPREPHO-4*. Transformants containing correctly oriented plasmid were identified by restriction analysis. A 5.5kb KpnI fragment containing the GA promoter and the pH2.5 Acid phosphatase gene was removed from *pPREPHO-4* by partial digestion. The resulting fragment was purified from agarose with GeneClean and blunt-ended with T4 DNA Polymerase. The blunt-ended fragment was ligated into *pLO-3* cut with PstI, and then the DNA was blunt-ended to give plasmids in two different orientations, termed *pPHO-4A* and *pPHO-4B*. Transformants containing each orientation were identified by restriction analysis. Linear DNA was isolated as an 8.1kb HindIII fragment.

DNA transformation of *A. niger* strains ALKO243 and ALKO2268:

[0147] Spheroplasts were obtained by first adding 0.5ml to 1ml of a fungal spore suspension from conidiating cultures grown on PD slants to 50ml of CM media in 250-ml flasks. For ALKO243, cultures were grown overnight at 35°C, 200 RPM, before filtration. ALKO2268 cultures were grown 48 hours at 30°C, 200 RPM. The resulting mycelia were collected onto a double layer of cheesecloth, added to 50ml of KCM buffer (0.7M KCl, 10mM MOPS, pH5.8) with 5mg/ml Novozym 234 (Novo BioLabs) and incubated at 30°C, 85 RPM overnight for spheroplast generation.

[0148] The spheroplasts were harvested by filtration through a funnel packed with mira cloth and covered with cheesecloth into four 15ml conical centrifuge tubes, then spun for ten minutes, 1500 RPM in a bench top centrifuge. The pellets were gently resuspended in a total of 15ml Sorbitol Buffer (1M Sorbitol, 50mM CaCl₂) and re-centrifuged. The pellet was again washed in Sorbitol Buffer (SB) then resuspended in SB to a density of 5 x 10⁷/ml.

[0149] 5 µg of linear or plasmid DNA in 20 µl TE was added to 200 µl of spheroplasts. For co-transformations 1 µg of selectable phleomycin resistance marker gene DNA, *pLO3*, was also added. 50 µl of PCM (40% PEG 8000, 10mM MOPS, pH5.8, 50mM CaCl₂ [CaCl₂ added just before use]) was gently pipetted into the DNA-spheroplast mixture and incubated on ice for 30 minutes.

[0150] 1ml of PCM was added to the transformation mix, the mix was pipetted into 50ml of Regeneration Agar (MA: CM plus 1.3M mannitol, 3% agar) and divided into five petri dishes. Spheroplasts were allowed to regenerate 3 to 5 hours at 35°C before overlaying with an equal amount of OL + phleomycin (OL: 1% peptone, 1% agar; phleomycin [CAYLA]: 50 µg/ml for ALKO243, 195 µg/ml for ALKO2268). Putative transformants were transferred to PD + phleomycin slants and grown at 28°C.

Phytase plate assay:

[0151] Transformants producing higher phytase yields were identified by a plate assay that colorimetrically measures the accumulation of inorganic phosphate by the reduction of a phosphomolybdate complex. Conidia from putative transformants was spotted onto assay plates and incubated two days at 30°C. 20ml of Reagent C (3:1:1 ratio of 1M sulfuric acid [Mallinckrodt], 2.5% ammonium molybdate [Sigma], 10% ascorbic acid [Sigma]) was applied to the top of the media, and the plates were incubated at 50°C for fifteen minutes before scoring for color intensity. (Plate assay agar: 2% corn starch [Sigma #S-4126], 1% protease peptone [Difco #0122-1], 30g glucose/l, 5g NH₄NO₃/l, 0.5g MgSO₄•7H₂O/l, 0.5g KCl, 0.183g FeSO₄•7H₂O/l, 3% agar, 3% sodium phytate [Sigma #P-3168].)

Phytase and pH2.5 acid phosphatase enzyme assays:

[0152] Enzyme assays were conducted as described in Example 1, above.

Production of phytase and pH2.5 acid phosphatase by transformed recombinant cells:

[0153] Transformed recombinant filamentous fungi expressing phytase and pH2.5 acid phosphatase were cultivated under identical conditions in a soy-based production medium [(50g/l soy flour, 30g/l glucose, 5g/l NH₄NO₃, 0.5g/l MgSO₄•7H₂O, 0.5g/l KCl, 0.183g/l FeSO₄•7H₂O, pH5.0)] for five days on a rotary shaker at 200 RPM and 28°C. Transformants utilizing the glucoamylase promoter were additionally grown in soy glucoamylase media (soy media plus 4% corn starch and 6% glucose. Enzyme samples were collected by centrifuging an aliquot of the fermentation culture at 13,000 RPM in a microcentrifuge for ten minutes, then transferring the enzyme-containing supernatant to a fresh tube for enzyme assay (below).

EXAMPLE 5Balanced over-expression of both the phytase gene and the pH2.5 acid phosphatase geneAnalysis of dual-enzyme-transformants with both phytase and pH2.5 AP genes:

[0154] Because both pH2.5 acid phosphatase and phytase are required for optimal degradation of phytic acid, it was considered highly advantageous to construct strains that might over-produce both enzymes. Since phytic acid was dephosphorylated most efficiently when ratios of phytase units to acid phosphatase units are tailor-made for the specific use (Example 1, above), it was considered even more desirable if transformant strains could be selected that would over-produce both enzymes in this desired range of ratio.

[0155] *A. niger* ALKO243 transformants were selected using the combinations of transformation vector plasmids shown in Table 13 and co-transfection with a phleomycin selectable marker (as described above, Example 4, *Materials*

and Methods). Following co-transformation with the indicated combinations of phytase and pH2.5 acid phosphatase containing plasmids (Table 13), the transformants were grown in shaker flask cultures (as described in Table 7 and Table 10, Example 4, above) to evaluate enzyme production.

[0156] All dual-enzyme transformants were first assayed to determine the amount of pH2.5 acid phosphatase; if levels were significantly elevated, then phytase activity was assayed. (Since phytase has two pH optimums at pH2.5 and pH5.0 [above, Example 1], its activity can be detected under pH2.5 acid phosphatase parameters.) (The acid phosphatase values were adjusted to account for the percentage of phytase activity that appears in the pH2.5 acid phosphatase assay.) Nine of 425 transformants were found to over-produce both phytase and pH2.5 acid phosphatase; and, at greater than 4:1 HFU/PU (i.e., pH2.5 acid phosphatase HFU divided by phytase PU)

TABLE 13

DNA Constructs Used to Construct Dual-Enzyme-Transformants of ALKO243 with Elevated Levels of both pH2.5 Acid Phosphatase and Phytase	
Plasmid DNA Combination	Number Analyzed
<i>pPHO1/pFF4</i>	3
<i>pPHO3/pFF1</i>	108
<i>pPHO3/pFF2</i>	50
<i>pPHO3/pFF4</i>	11
<i>pPHO3(lin)/pFF6(lin)</i>	74
<i>pPHO3(lin)/pFFB(lin)</i>	7
<i>pPHO4A/pFF1</i>	21
<i>pPHO4A/pFF2</i>	22
<i>pPHO4A/pFF3</i>	25
<i>pPHO4A/pFF4</i>	22
<i>pFF6/prepho2</i>	2
<i>pFIN-1A</i>	84
<i>pFIN-1B</i>	46
Total	475

[0157] Eighteen of the 475 combination transformants (Table 13) demonstrated activities in excess of 20-fold HFU/ml greater than ALKO243 for pH2.5 acid phosphatase and 250-fold PU/ml greater than ALKO243 for phytase activity (Table 14).

TABLE 14

<i>A. niger</i> ALKO243 Dual-Enzyme-Transformants Expressing Both Phytase and pH2.5 Acid Phosphatase (pH2.5AP) at Levels of Enzyme Activity Greater than the ALKO243 parent cell by greater than 20-fold for pH2.5 Acid Phosphatase (A.P.) and greater than 250-fold for Phytase (Phy.) ^a						
Transformant	Plasmid(s)/ Promoter AP/Phy	Activity HFU/ml	Activity PU/ml	Ratio HFU/PU	Increase A.P.	Increase Phytase
GAX-11	<i>pPHO-4A/</i> <i>pFF-4 (GA/</i> <i>GA)</i>	280,500	53,000	5.3	51	624

a.) Enzyme activities were determined in shake flask cultures (as described in Table 10, Example 4, above). The results are expressed as the average of at least two independent fermentation cultures except (*) which was taken from a single fermentation culture. pH2.5 acid phosphatase (pH2.5 AP) levels were adjusted to subtract the background phytase activity (i.e., the percentage of phytase that could be detected at pH2.5 in the pH2.5 acid phosphatase assay conditions; *Materials and Methods*); b.) All plasmids utilized in these studies were circular, and in the whole circular form. Plasmids *pFIN-1A* and *pFIN-1B* are single plasmids that contain both the pH2.5 acid phosphatase (A.P.) and phytase (Phy.) gene sequence. Transformant = Transformant designation; (Promoter for acid phosphatase (A.P.)/promoter for phytase (Phy)); GA = glucoamylase promoter; GAPDH = glyceraldehyde 3-phosphate dehydrogenase promoter; β -T = β -Tubulin promoter; c.) HFU/ml, acid phosphatase units; PU/ml, phytase units (see Tables 7-10, above); d.) Ratio HFU/PU = Activity for acid phosphatase (HFU/ml)/Activity for phytase (PU/ml); e.) Increase is expressed as the fold increase in acid phosphatase (HFU/ml) or phytase (PU/ml) activity in shake flask culture over the activity with ALKO243 in shake flask culture (i.e., activity in HFU/ml or PU/ml, respectively, of transformant per activity in HFU/ml or PU/ml, respectively, of ALKO243).

TABLE 14 (continued)

A. niger ALKO243 Dual-Enzyme-Transformants Expressing Both Phytase and pH2.5 Acid Phosphatase (pH2.5AP) at Levels of Enzyme Activity Greater than the ALKO243 parent cell by greater than 20-fold for pH2.5 Acid Phosphatase (A.P.) and greater than 250-fold for Phytase (Phy.)^a

Transformant	Plasmid(s)/ Promoter AP/Phy	Activity HFU/ml	Activity PU/ ml	Ratio HFU/ PU	Increase A.P.	Increase Phytase
GAX-12	<i>pPHO-4A/</i> <i>pFF-4</i> (GA/ GA)	176,000	29,000	6.1	32	342
GBE-14	<i>pPHO-3/</i> <i>pFF-4</i> (GAPDH/ GA)	147,300	29,400	5.0	27	346
GBH-134	<i>pPHO-3/</i> <i>pFF-2</i> (GAPDH/ β-T)	291,500	39,000	7.5	53	459
GBH-157	<i>pPHO-3/</i> <i>pFF-2</i> (GAPDH/ β-T)	247,500	54,000	4.6	45	635
GBJ-9	<i>pFIN-1A</i> (GA/GA)	212,400	38,800	5.5	39*	456
GBJ-10	<i>pFIN-1A</i> (GA/GA)	241,000	38,100	5.6	44*	448
GBJ-13	<i>pFIN-1A</i> (GA/GA)	259,800	25,300	10.2	47*	299
GBJ-16	<i>pFIN-1A</i> (GA/GA)	280,600	23,200	12.1	51*	273
GBJ-26	<i>pFIN-1B</i> (GA/GA)	328,100	45,700	7.2	60*	538
GBJ-27	<i>pFIN-1B</i> (GA/GA)	218,100	39,700	5.5	40*	467
GBJ-28	<i>pFIN-1B</i> (GA/GA)	372,300	23,600	15.8	68*	278
GBJ-31	<i>pFIN-1B</i> (GA/GA)	299,000	30,400	9.8	54*	358
GBJ-35	<i>pFIN-1B</i> (GA/GA)	255,300	28,400	9.0	46*	334
GBJ-38	<i>pFIN-1B</i> (GA/GA)	277,500	25,600	10.8	50*	301
GBJ-40	<i>pFIN-4A</i> (GA/GA)	401,000	38,200	10.5	73	449

a.) Enzyme activities were determined in shake flask cultures (as described in Table 10, Example 4, above). The results are expressed as the average of at least two independent fermentation cultures except (*) which was taken from a single fermentation culture. pH2.5 acid phosphatase (pH2.5 AP) levels were adjusted to subtract the background phytase activity (i.e., the percentage of phytase that could be detected at pH2.5 in the pH2.5 acid phosphatase assay conditions; *Materials and Methods*); b.) All plasmids utilized in these studies were circular, and in the whole circular form. Plasmids *pFIN-1A* and *pFIN-1B* are single plasmids that contain both the pH2.5 acid phosphatase (A.P.) and phytase (Phy.) gene sequence. Transformant = Transformant designation; (Promoter for acid phosphatase (A.P.)/promoter for phytase (Phy)); GA = glucoamylase promoter; GAPDH = glyceraldehyde 3-phosphate dehydrogenase promoter; β-T = β-Tubulin promoter; c.) HFU/ml, acid phosphatase units; PU/ml, phytase units (see Tables 7-10, above); d.) Ratio HFU/PU= Activity for acid phosphatase (HFU/ml)/Activity for phytase (PU/ml); e.) Increase is expressed as the fold increase in acid phosphatase (HFU/ml) or phytase (PU/ml) activity in shake flask culture over the activity with ALKO243 in shake flask culture (i.e., activity in HFU/ml or PU/ml, respectively, of transformant per activity in HFU/ml or PU/ml, respectively, of ALKO243).

TABLE 14 (continued)

A. niger ALKO243 Dual-Enzyme-Transformants Expressing Both Phytase and pH2.5 Acid Phosphatase (pH2.5AP) at Levels of Enzyme Activity Greater than the ALKO243 parent cell by greater than 20-fold for pH2.5 Acid Phosphatase (A.P.) and greater than 250-fold for Phytase (Phy.)^a

Transformant	Plasmid(s)/ Promoter AP/Phy	Activity HFU/ml	Activity PU/ ml	Ratio HFU/ PU	Increase A.P.	Increase Phytase
GBJ-76	<i>pPHO-4A/ pFF-2 (GA/ β-T</i>	201,600	46,100	4.4	37*	542
GBJ-82	<i>pPHO-4A/ pFF-2 (GA/ β-T)</i>	309,800	40,100	7.7	56*	472
ALKO243	control/ native (native)	5,500	85	64.7	1	1

a.) Enzyme activities were determined in shake flask cultures (as described in Table 10, Example 4, above). The results are expressed as the average of at least two independent fermentation cultures except (*) which was taken from a single fermentation culture. pH2.5 acid phosphatase (pH2.5 AP) levels were adjusted to subtract the background phytase activity (i.e., the percentage of phytase that could be detected at pH2.5 in the pH2.5 acid phosphatase assay conditions; *Materials and Methods*); b.) All plasmids utilized in these studies were circular, and in the whole circular form. Plasmids *pFIN-1A* and *pFIN-1B* are single plasmids that contain both the pH2.5 acid phosphatase (A.P.) and phytase (Phy.) gene sequence. Transformant = Transformant designation; (Promoter for acid phosphatase (A.P.)/promoter for phytase (Phy)); GA = glucoamylase promoter; GAPDH = glyceraldehyde 3-phosphate dehydrogenase promoter; β-T = β-Tubulin promoter; c.) HFU/ml, acid phosphatase units; PU/ml, phytase units (see Tables 7-10, above); d.) Ratio HFU/PU = Activity for acid phosphatase (HFU/ml)/Activity for phytase (PU/ml); e.) Increase is expressed as the fold increase in acid phosphatase (HFU/ml) or phytase (PU/ml) activity in shake flask culture over the activity with ALKO243 in shake flask culture (i.e., activity in HFU/ml or PU/ml, respectively, of transformant per activity in HFU/ml or PU/ml, respectively, of ALKO243).

Molecular Characterization:

[0158] Strains GAX7, GAX-11 and GAX-12 were characterized to determine their relative amounts of gene dosage, message levels and enzyme production. The three transformants all show evidence of tandem integration occurring from the introduction of the phytase transformation vector *pFF4*. Two of the strains, GAX-7 and GAX-11, also show evidence of multiple integration events (FIGURE 17A). The increase in phytase message level over ALKO243 mRNA levels varied from a 17.5-fold increase (for GAX-11) to a 34.4-fold increase (for GAX-12) (FIGURE 17B; Table 15).

FIGURE 17A shows Southern analysis of DNA copy number of phytase genes in the dual-gene-transformed GAX strains. DNA from dual-enzyme-transformants GAX-7, 11, and 12 was digested with BamHI and XbaI; electrophoresed; blotted; and, the filter was probed with a BamHI/XbaI fragment from *pFF-4*. Lane 1. *pFF-4* plasmid control DNA digested with BamHI/XbaI. Lane 2. ALKO243 untransformed control DNA. Lane 3. Transformant GAX-7 DNA. Lane 4. Transformant GAX-11 DNA. Lane 5. Transformant GAX-12 DNA. p = transformation vector plasmid copy number; c = chromosomal gene copy number.

FIGURE 17B shows Northern analysis of phytase mRNA transcript levels in the GAX strains of FIGURE 17A: 20mg of total RNA, spiked with 10ng of mp 18 RF DNA (mp) was electrophoresed, blotted to nitrocellulose, and probed with *pFF-1*. The phytase transcript is arrowed at 1.4kb. Lane 1. ALKO243 untransformed control RNA. Lane 2. GAX-7 RNA. Lane 3. GAX-11 RNA. Lane 4. GAX-12 RNA.

TABLE 15

Comparison of Phytase Copy Number, mRNA Levels, and Enzyme Activity for Dual-Enzyme-Transformants ^a					
Strain	Plasmid	Vector Copy No. ^b	mRNA ^c	Activity ^d	Increase ^e
ALKO243	Alone 1	1.0	1.0	85	1
GAX-7	<i>pFF4</i> (GA, cir)	2 ⁺	24.4	38,400	452
GAX-11	<i>pFF4</i> (GA, cir)	6 ⁺	17.5	53,000	624
GAX-12	<i>pFF4</i> (GA, cir)	8	34.4	29,000	341

a.) GA = glucoamylase promoter; cir = circular vector;

b.) Vector copy no. = the number of copies of the vector nucleic acid in the genome of the transformed cell; + = unquantitated additional copies of phytase present;

c.) mRNA = messenger RNA amplification levels, (as fold-increase over levels in ALKO243);

d.) Activity = phytase activity represented in PU/ml from shake flask cultures as described in Table 10. above;

e.) Increase is expressed as the fold increase in phytase (PU/ml) activity in shake flask culture over the activity with ALKO243 in shake flask culture (i.e., activity in PU/ml, respectively, of transformant per activity in PU/ml, respectively, of ALKO243).

[0159] Data from a similar analysis by Southern and Northern blotting of the three strains are presented in Table 16, below, for pH2.5 acid phosphatase transformation vector gene sequences. The results show that GAX-7, a *pPHO-3* integrant, contained one extra copy of pH2.5 acid phosphatase (as a single integration event); whereas GAX-11 and 12, *pPHO-4A* integrants, contained extra copies (acquired by tandem integration; FIGURE 18A). Message levels for pH2.5 acid phosphatase were increased 2.7-fold (i.e., over levels in ALKO243) in GAX-7, and 10-fold and 15-fold in GAX-11 and GAX-12, respectively (FIGURE 18B; Table 16). As observed for the single pH2.5 acid phosphatase gene transformants (i.e., Example 4 above), higher copy numbers were obtained for integration of transformation vector plasmids than with the phytase vectors, but the message levels for pH2.5 acid phosphatase were not corresponding as high as the levels observed with the phytase vectors (Table 16, below).

FIGURE 18A shows Southern analysis of DNA copy number of pH2.5 acid phosphatase sequences in GAX dual-gene-transformed strains. All samples were digested with *Sall*, electrophoresed, blotted to nitrocellulose, and probed with the pH2.5 acid phosphatase gene as isolated in fragments from *pAP-3* (Example 3, above). Lane 1. *pPHO-3* plasmid DNA control, *Sall* digested. Lane 2. *pPHO-4A* plasmid DNA control, *Sall* digested. Lane 3. Untransformed ALKO243 DNA control. Lane 4. GAX-7 DNA. Lane 5. GAX-11 DNA. Lane 6. GAX-12 DNA.

FIGURE 18B shows Northern analysis of mRNA levels of pH2.5 acid phosphatase message expression in dual-enzyme transformed in strains. The pH2.5 acid phosphatase RNA transcript is arrowed. Lane 1. ALKO243 untransformed control RNA. Lane 2. GAX-7 RNA. Lane 3. RNA. GAX-11 RNA. Lane 4. GAX-12 RNA.

TABLE 16

Comparison of pH2.5 acid phosphatase copy number, mRNA Levels, and Enzyme Activity in Dual-Enzyme-Transformants ^a					
Strain	Plasmid	Vector Copy No. ^b	mRNA ^c	Activity ^d	Increase ^e
ALKO243	---	1.0	1	5,500	1.0
GAX-7	<i>pPHO3</i> (GAP, cir)	1	2.7	112,100	20
GAX-11	<i>pPHO4A</i> (GA, cir) 16		10.3	280,500	51
GAX-12	<i>pPHO4A</i> (GA, cir)	16	16.0	176,000	32

a.) GAP = glyceraldehyde 3-phosphate dehydrogenase promoter; GA = glucoamylase promoter; cir = circular vector;

b.) Vector copy no., see Table 15, above;

c.) mRNA = messenger RNA amplification levels, (as fold-increase over Levels in ALKO243);

d.) Activity = pH2.5 acid phosphatase activity represented in HFU/ml measured in shake flask cultures as described in Table 10, above;

e.) Increase is expressed as the fold increase in acid phosphatase (HFU/ml) activity in shake flask culture over the activity with ALKO243 in shake flask culture (i.e., activity in HFU/m) of transformant per activity in HFU/ml of ALKO243).

Materials and Methods:

[0160] The materials and methods used herein were the same as those used above (see Example 4).

EXAMPLE 6Control of over-expression levels of phytase and pH2.5 acid phosphatase in dual-transformants

[0161] As described above in Examples 2-5, industrially significant increases in the yields of phytase and pH2.5 acid phosphatase were achieved by transforming a strain of *Aspergillus niger* (i.e., ALKO2268) with transformation vector constructs and selecting for transformants that over-expressed the desired enzymatically active protein. In shake flask fermentations, phytase yields were increased 2,000-fold over enzyme production levels achieved with ALKO243 (Example 4; Table 8, above), and pH2.5 acid phosphatase yields were increased more than 100-fold (Example 4; Table 11, above). These yield increases were considered to result from several factors: most importantly, increased gene dosage, and utilization of effective alternative promoters (i.e., heterologous promoters GA, GAP, GAPDH and the β -tubulin promoter; see Examples 4 and 5, above). Factors controlling expression levels were evaluated further.

The effect of gene dosage on fermentation levels of phytase and acid phosphatase:

[0162] The data presented in the Examples 4 and 5 above suggest that relatively large increases in phytase protein resulted from relatively modest increases in gene copy number and mRNA transcript levels. The observed gene copy number increases ranged from three to five additional copies in the phytase over-producing strains. These strains, namely GAE-32, GAK-47, GAL-65, GAM-225 and GAO-248, apparently acquired the additional gene copies by multiple integration events of the recombinant transformation vector plasmid DNA, i.e., rather than by tandem integration. Three of the five strains, GAL-65, GAM-225 and GAO-268, were transformed with linear DNA fragments, from which tandem arrays may be less likely to occur. GAE-32 and GAK-47, however, were transformed with circular vector constructions, which often give rise to tandemly arranged gene copies. Since the transformants were selected on the basis of phytase activity in the enzyme-plate assay (rather than by Southern blot analysis, as is commonly the case), the relative frequency of transformants that had integrated phytase DNA sequences in optimal locations was likely increased. Chromosomal integration site(s) apparently play an important role in phytase gene expression and this screening method selected optimized transformants.

[0163] In high producing phytase transformants, messenger RNA levels were elevated seven- to thirteen-fold over ALKO2268, while fermentation levels produced by the cells increased fifty-fold.

[0164] The copy number observed in pH2.5 acid phosphatase transformants (Table 12, Example 4; Table 16, Example 5, above) was higher than in phytase transformants (Table 9, Example 4; Table 15, Example 5, above), but the amounts of pH2.5 acid phosphatase message was substantially lower than in phytase transformants. High pH2.5 acid phosphatase over-producer strains GAT-143, GAW-54, GAW-89, GAW-121 and GAW-130 were transformed with vectors having linear DNA from either *pPHO-3* or *pPHO-4A*. Seven to twelve additional gene copies (i.e., vector gene copies) were seen in these transformants, but message levels were only increased by about three- to about seven-fold.

Effects of linear and circular DNA on titers of transformants:

[0165] The effects of linear DNA transformation vector constructs and circular vector constructs on phytase enzyme production were evaluated. Overall, circular constructions gave a higher frequency of transformants with increased phytase enzyme production (FIGURE 19), but the maximal levels of enzyme produced by the transformants were comparable with linear and circular constructions. For example, circular vector *pFF3* gave 8/24 (33%) phytase transformants with enzyme production levels greater than 750PU. The linear counterpart of *pFF3*, i.e., *pFF8*, had two-fold fewer transformants (i.e., that achieved the same threshold level of phytase production), but the transformants that were selected had maximal production levels comparable with *pFF8* transformants. When linear fragments were purified and religated, the frequency of higher producers did not increase.

FIGURE 19. Comparison of the effects of linear and circular DNA on the titers of twenty-four randomly selected phytase transformants. With the circular vector *pFF3*, 33% of the phytase transformants had levels of enzyme production greater than 750PNU. Its linear counterpart, *pFF8*, had two-fold fewer transformants that achieved that same threshold of enzyme production. PNU = phytase normalized units.

Effects of fungal promoters on product titers:

[0166] The level of expression of genes in *Aspergillus* may be dependent upon the particular choice of *A. niger*

filamentous fungal promoter. The results presented in Examples 4 and 5, above, support the notion that phytase and pH2.5 acid phosphatase can be expressed from a variety of *A. niger* promoters (e.g., including the GA, GAPDH and β -tubulin promoter), as well as the native promoter elements in the 5' regulatory regions of the phytase and pH2.5 acid phosphatase genes. However, the results also show that the maximal levels of enzyme produced in transformants, from expression directed by the different promoters, is different; i.e., the choice of promoter determined the level of expression. While the native phytase and pH2.5 acid phosphatase promoters were effective, the glucoamylase (GA) and GAPDH promoters were significantly better. Production levels of phytase and pH2.5 acid phosphatase in fermentation culture under the control of the GA and GAPDH promoters are shown in FIGURE 20.

FIGURE 20. Different fungal promoters used in transforming plasmids influenced the level of phytase and pH2.5 acid phosphatase produced by *A. niger* transformants. The levels of enzyme produced by ten transformants (i.e., with the highest levels for that given promoter), were averaged together. (Data from dual-enzyme-transformants were not used in these calculations.) GAPDH = glyceraldehyde 3-phosphate dehydrogenase. GA = glucoamylase.

[0167] The glucoamylase promoter from *Aspergillus* species has been reportedly used to increase expression of several proteins (Ullah et al., 1987). In addition to the GA promoter, the GA sequence contains a glucoamylase signal peptide and propeptide that have been reportedly used in attempts to increase expression (Yamamoto et al., 1970).

[0168] To test the effects of the phytase signal sequence on the levels of phytase produced, the signal sequence in the phytase gene was replaced (in construct *pFF-11*) by a synthetic oligonucleotide containing the fungal glucoamylase putative signal sequence. Surprisingly, instead of increasing the levels of enzyme produced, the insertion of the GA signal sequence actually decreased the levels of phytase produced, as compared with the levels of production achieved in transformants having the native phytase signal sequence (i.e., in the native phytase gene sequence). These results indicate the possible importance of phytase signal sequence in maximizing the levels of production of phytase.

[0169] Genes under the control of a glucoamylase promoter may be upregulated (induced) in the presence of starch. To evaluate the effects of such upregulation on levels of phytase production, some *pFF-4* and *pFF-9* glucoamylase-promoter phytase transformants were cultured in media that had been supplemented with 4% corn starch. Levels of enzyme production were increased an average of 1.4-fold in the latter cultures, although these levels of production were not consistently observed. The highest yields of phytase seen under inducing conditions were 3240 - 3770PNU. The results indicate that optimization of the media used in the phytase production fermentation broth could result in significantly greater yields of enzyme.

[0170] The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter is reportedly a constitutive promoter that may drive high-level expression of a heterologous gene product. The inventors independently cloned the glyceraldehyde phosphate dehydrogenase A gene (*gpdA*) from *A. niger* ATCC1015 in order to evaluate its effect on phytase and pH2.5 acid phosphatase gene expression in a transformation vector construct introduced into *A. niger* strain ALKO243 or ALKO2268. The levels of enzyme production in transformants having the GAPDH promoter driving expression of phytase and pH2.5 acid phosphatase were nearly identical to the levels achieved, above, using the glucoamylase promoter (FIGURE 20). Thus, the GAPDH promoter did not appear to offer any unique opportunities for increased enzyme production. The reasons for this particular behavior are presently unclear; however, such variability in expression between ALKO243 and ALKO2268 may relate to the derivation of ALKO2268 as a mutant from ALKO243, and in this regard ALKO2268 produces only about one-half of the amount of pH2.5 acid phosphatase produced by the parental ALKO243 strain. Thus, it is possible that ALKO2268 is deficient in some limiting factors relating to expression of pH2.5 acid phosphatase. (Such strain-dependent differences in promoter-driven expression were not observed with any other promoters.)

[0171] The β -tubulin promoter is also reportedly a promoter capable of driving constitutive expression of genes. To tests its possible effects on phytase and pH2.5 acid phosphatase gene expression, the β -tubulin promoter was isolated from *A. niger* strain 1015. The promoter was introduced into transformation vectors to evaluate its effects on the levels of phytase and pH2.5 acid phosphatase production. Previous studies by the inventors suggested that expression of homologous fungal genes could be increased when driven by the β -tubulin promoter, and that this increase was substantially greater than that which could be achieved through the use of a GAPDH- or GA-promoter (Panlabs, unpublished). Unfortunately, inclusion of a β -tubulin promoter in the phytase transformation vector construct actually decreased the levels of enzyme produced; and, with pH2.5 acid phosphatase only a modest increase in production levels was noted over the levels of production mediated by the native promoter (FIGURE 20).

[0172] It was also considered possible that production of recombinant phytase and pH2.5 acid phosphatase might be sensitive to phosphate-mediated repression of transcription. In this case, the levels of enzyme produced might be increased by eliminating such conditions in the fermentation culture. In support of the notion of phosphate-mediated repression, cells of both the ALKO243 and ALKO2268 strains produced no detectable phytase when excess phosphate was added to the fermentation culture broth. To study this possibility further, the levels of enzyme produced by phytase transformants having alternative promoters (i.e., GA, GAPDH or β -tubulin) were compared to the levels achieved with the native phytase and pH2.5 acid phosphatase promoters in the transformation vector constructs to see if the use of such an alternative promoter eliminated the putative phosphate-mediated repression. Interestingly, no phytase pro-

duction was detected in either the phytase or the pH2.5 acid phosphatase promoter when the transformants were grown in the presence of phosphate, supporting the notion of phosphate-mediated repression that cannot be overcome through use of the native promoter integrated into a different chromosomal site. In evaluating the alternative promoters, one transformant with a β -tubulin promoter was able to produce phytase in the presence of phosphate, but it showed a decrease in phytase production by about one-half. In contrast, GAPDH-promoter and GA-promoter transformants were not sensitive to phosphate-mediated inhibition to any degree, and yielded similar levels of phytase production in the presence or absence of additional phosphate. Thus, the results suggest that regulatory elements in the 5' regulatory region of the phytase and pH2.5 acid phosphatase genes may be useful for directing phosphate-mediated repression of gene expression, and that insertion of alternative promoters into the 5' regions of these genes can overcome these native regulatory constraints and increase production yields.

[0173] The identification of rate limiting factors, whether energy or secretion related, may further increase yields of phytase and pH2.5 acid phosphatase. Other factors such as media optimization, increased or stabilized gene transcription levels of pH2.5 acid phosphatase, or classical mutagenesis screening of high producers could have additional impacts on product yield. Elevated gene dosage and the use of alternative promoters have dramatically increased production yields over native promoters and escaped negative regulatory constraints. The cloning and reintroduction of the genes for phytase and pH2.5 acid phosphatase has resulted in commercially significant levels of these enzymes for use as animal feed supplements. Rational design of homologous gene over-expression can be applied to increase the yields of other fungal industrial products, and potentially give a better understanding of the mechanisms required for heterologous expression.

Materials and Methods:

[0174] The materials and methods used in this example are the same as those used in Example 4, above.

CITATIONS

[0175]

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

[0176]

(i) APPLICANT:

- (A) NAME: Alko Group Ltd.
- (B) STREET: Salmisaarenranta 7
- (C) CITY: Helsinki
- (D) COUNTRY: Finland
- (E) POSTAL CODE (ZIP): FIN-00180

(ii) TITLE OF INVENTION: "Recombinant Cells, DNA Constructs, Vectors and Methods For Expressing Phytate Degrading Enzymes in Desired Ratios

(iii) NUMBER OF SEQUENCES: 47

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: WordPerfect 5.1

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: PCT/US93/07058

(2) INFORMATION FOR SEQ ID NO : 1 :

[0177]

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(ix) FEATURE:

OTHER INFORMATION: Peptide #420

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Leu Tyr Val Glu Met Met Gln Cys Gln Ala Glu Gln
 1 5 10

(3) INFORMATION FOR SEQ ID NO:2:

[0178]

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(ix) FEATURE:

OTHER INFORMATION: Peptide #816C

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Arg His Gly Glu Arg Tyr Pro Ser Pro Ser Ala Gly Lys
 1 5 10

(4) INFORMATION FOR SEQ ID NO:3:

[0179]

5 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(ix) FEATURE:

15 OTHER INFORMATION: Peptide #1110T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

20 Gln Leu Pro Gln Phe Lys
 1 5

25 (5) INFORMATION FOR SEQ ID NO:4:

[0180]

30 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 bases
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURE:

40 OTHER INFORMATION: Oligo PHY-1; read 5' to 3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

45 GCTTGGCATT GTACTAC

17

(6) INFORMATION FOR SEQ ID NO:5:

50 **[0181]**

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 bases
 55 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURE:

5 OTHER INFORMATION: Oligo PHY-1; read 5' to 3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

10 GCCTGACACT GTACTAC

17

(7) INFORMATION FOR SEQ ID NO:6:

15 **[0182]**

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 17 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

25

(ix) FEATURE:

30 (A) NAME/KEY: misc-feature
(B) LOCATION: 6
(D) OTHER INFORMATION: N is dIMP

(ix) FEATURE:

35 OTHER INFORMATION: Oligo PHY-31; read 5' to 3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

40 GGGTANCGCT CGCCGTG

17

(8) INFORMATION FOR SEQ ID NO:7:

[0183]

45

(i) SEQUENCE CHARACTERISTICS:

50 (A) LENGTH: 17 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

55

(ix) FEATURE:

(A) NAME/KEY: misc-feature
(B) LOCATION: 6

(D) OTHER INFORMATION: N is dIMP

(ix) FEATURE:

5 OTHER INFORMATION: Oligo PHY-31; read 5' to 3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

10 GGATANCTTT CACCATG

17

(9) INFORMATION FOR SEQ ID NO:8:

15

[0184]

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 17 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURE:

30 (A) NAME/KEY: misc-feature
(B) LOCATION: 6
(D) OTHER INFORMATION: N is dIMP

(ix) FEATURE:

35 OTHER INFORMATION: Oligo PHY-31; read 5' to 3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

40 GGGTANCGCT CGCCGTG

17

(10) INFORMATION FOR SEQ ID NO:9:

[0185]

45

(i) SEQUENCE CHARACTERISTICS:

50 (A) LENGTH: 17 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

55 (ix) FEATURE:

(A) NAME/KEY: misc-feature
(B) LOCATION: 6

(D) OTHER INFORMATION: N is dIMP

(ix) FEATURE:

5 OTHER INFORMATION: Oligo PHY-31; read 5' to 3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

10 GGGTANCGCT CCCCCGTG

17

(11) INFORMATION FOR SEQ ID NO:10:

15 **[0186]**

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 17 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURE:

OTHER INFORMATION: Oligo PHY-34; read 5' to 3'

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CAACTGCCGC AATTTAA

17

35

(12) INFORMATION FOR SEQ ID NO:11:

[0187]

40 (i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 17 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURE:

50

OTHER INFORMATION: Oligo PHY-34; read 5' to 3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

55

CAGCTACCAC AGTTCAA

17

(13) INFORMATION FOR SEQ ID NO:12:

[0188]

5 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 bases
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURE:

15 OTHER INFORMATION: Oligo PHY-34; read 5' to 3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

20 CAACTTCCTC AATTTAA 17

(14) INFORMATION FOR SEQ ID NO:13:

[0189]

25 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 bases
 30 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

35 (ix) FEATURE:

OTHER INFORMATION: Oligo PHY-34; read 5' to 3'

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CAACTCCCCC AATTTAA 17

(15) INFORMATION FOR SEQ ID NO:14:

[0190]

50 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 bases
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 55 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURE:

OTHER INFORMATION: Oligo PHY-35; read 5' to 3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

5 CAATTACCGC AATTTAA 17

(16) INFORMATION FOR SEQ ID NO:15:

10 [0191]

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 17 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURE:

25 OTHER INFORMATION: Oligo PHY-35; read 5' to 3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

30 CAGTTGCCAC AGTTCAA 17

(17) INFORMATION FOR SEQ ID NO:16:

35 [0192]

(i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 17 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

45 (ix) FEATURE:

OTHER INFORMATION: Oligo PHY-35; read 5' to 3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

50 CAATTACCTC AATTTAA 17

(18) INFORMATION FOR SEQ ID NO:17:

55 [0193]

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 bases
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(ix) FEATURE:

OTHER INFORMATION: Oligo PHY-35; read 5' to 3'

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CAATTACCCC AATTTAA

17

(19) INFORMATION FOR SEQ ID NO:18:

[0194]

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2379 bases
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURE:

OTHER INFORMATION: Sequence of Figure 9

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TGATGGCGGC	CTAGGGCATC	CAGGCACCCT	TTCCCAACGG	GGGAACTTCC	GTTGTCCACG	60
TGCCCTGGTT	CAGCCAATCA	AAGCGTCCCA	CGGCAATGCT	GGATCAACGA	TCAACTTGAA	120
TGCAATAAAT	GAAGATGCAA	CTAACACCAT	CTGTTGCCCTT	TCTCTCGAGA	AAGCTCCTCC	180
ACTTCTCACA	CTAGATTTAT	CCGTTCCCTTG	TCGACTTCCC	GTCCCATTCG	GCCTCGTCCA	240
CTGAAGATCT	ATCCCACCAT	TGCACGTGGG	CCACCTTTGT	GAGCTTCTAA	CCTGAACTGG	300
TAGAGTATCA	CACAACATGC	GAAAGTGGGA	TGAAGGGGTT	ATATGAGGAC	CGTCCGGTCC	360
GGCGCGATGG	CCGTAGCTGC	CAATCGCTGC	TGTGCAAGAA	ATTTCTTCTC	ATAGGCATCA	420
TGGGCGTCTC	TGCTGTTCTA	CTTCCTTTGT	ATCTCCTAGC	TGGGTATGCT	AAGCACCGCT	480
ATCTAAGTCT	GATAAGGACC	CTCTTTGCCG	AGGGCCCCTG	AAGCTCGGAC	TGTGTGGGAC	540
TACTGATCGC	TGACAATCTG	TGCAGAGTCA	CCTCCGGA	GGCAGTCCCC	GCCTCGAGAA	600
ATCAATCCAC	TTGCGATACG	GTCGATCAAG	GGTATCAATG	CTTCTCCGAG	ACTTCGCATC	660
TTTGGGGTCA	ATACGCGCCG	TTCTTCTCTC	TGGCAAACGA	ATCGGCCATC	TCCCCTGATG	720
TGCCCGCCGG	TTGCAGAGTC	ACTTTCGCTC	AGGTCCCTCTC	CCGTCATGGA	GCGCGGTATC	780
CGACCGAGTC	CAAGGGCAAG	AAATACTCCG	CTCTCATTGA	GGAGATCCAG	CAGAACGTGA	840
CCACCTTTGA	TGGAAAATAT	GCCTTCCTGA	AGACATACAA	CTACAGCTTG	GGTGCAGATG	900
ACCTGACTCC	CTTCGGAGAG	CAGGAGCTAG	TCAACTCCGG	CATCAAGTTC	TACCAGCGAT	960
ACGAATCGCT	CACAAGGAAC	ATCATTCCGT	TCATCCGATC	CTCTGGCTCC	AGCCGCGTGA	1020
TCGCCTCCGG	CGAGAAATTC	ATTGAGGGCT	TCCAGAGCAC	CAAGCTGAAG	GATCCTCGTG	1080
CCCAGCCGGG	CCAATCGTCG	CCCAAGATCG	ACGTGGTTCAT	TTCCGAGGCC	AGCTCATCCA	1140
ACAACACTCT	CGACCCAGGC	ACCTGCACTG	TCTTTGAAGA	CAGCGAATTG	GCCGATACCG	1200

TCGAAGCCAA TTTCACCGCC ACGTTCGCCC CCTCCATTCTG TCAACGTCTG GAGAACGACC 1260
 TGTCTGGCGT GACTCTCACA GACACAGAAG TGACCTACCT CATGGACATG TGCTCCTTCG 1320
 ACACCATCTC CACCAGCACC GTCGACACCA AGCTGTCCCC CTTCTGTGAC CTGTTCAACC 1380
 5 ATGACGAATG GATCCACTAC GACTACCTCC AGTCCCTGAA AAAATACTAC GGCCATGGCG 1440
 CAGGTAACCC GCTCGGCCCG ACCCAGGGCG TCGGCTACGC TAACGAGCTC ATCGCCCGTC 1500
 TCACCCACTC GCCTGTCCAC GATGACACCA GCTCCAACCA CACCTTGGAC TCGAACCCAG 1560
 CTACCTTCCC GCTCAACTCT ACTCTCTACG CGGACTTTTC CCACGATAAC GGCATCATCT 1620
 CTATCCTCTT TGCTTTGGGT CTGTACAACG GCACTAAGCC GCTGTCTACC ACGACCGTGG 1680
 AGAATATCAC CCAGACAGAT GGGTTCTCGT CTGCTTGGAC GGTTCGTTT GCTTCGCGTC 1740
 10 TGTACGTCGA GATGATGCAG TGCCAGGCCG AGCAGGAGCC GCTGGTCCGT GTCTTGTTA 1800
 ATGATCGCGT TGTCCCCTG CATGGGTGTC CAATTGATGC TTTGGGGAGA TGTACCCGGG 1860
 ATAGCTTTGT GAGGGGGTTG AGCTTTGCTA GATCTGGGGG TGATTGGGCG GAGTGTTCG 1920
 CTTAGCTGAA CTACCTTGAT GGATGGTATG TATCAATCAG AGTACATATC ATTACTTCAT 1980
 GTATGTATTT ACGAAGATGT ACATATCGAA ATATCGATGA TGACTACTCC GGTAGATATT 2040
 TGGTCCCCTT CTATCCTTCG TTCCACAACC ATCGCACTCG ACGTACAGCA TAATACAACT 2100
 15 TCAGCATTA ACAAACGAACA AATAATATTA TACACTCCTC CCCAATGCAA TAACAACCGC 2160
 AATTTCATAC TCATATAGAT ACAATACAAT ACATCCATCC CTACCCTCAA GTCCACCCAT 2220
 CCCATAATCA AATCCCTACT TACTCCTCCC CCTTCCCAGA ACCCACCCTC GAAGGAGTAA 2280
 TAGTAGTAGT AGAAGAAGCA GACGACCTCT CCACCAACCT CTTCCGGCCTC TTATCCCCAT 2340
 ACGCTATACA CACACGAACA CACCAAATAG TCAGCATGC 2379

20

(20) INFORMATION FOR SEQ ID NO:19:

[0195]

25 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 467 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE

35

OTHER INFORMATION: Protein encoded by SEQ ID NO:18

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

40

Met Gly Val Ser Ala Val Leu Leu Pro Leu Tyr Leu Leu Ala Gly Val
 1 5 10 15

Thr Ser Gly Leu Ala Val Pro Ala Ser Arg Asn Gln Ser Thr Cys Asp
 20 25 30

45

Thr Val Asp Gln Gly Tyr Gln Cys Phe Ser Glu Thr Ser His Leu Trp
 35 40 45

Gly Gln Tyr Ala Pro Phe Phe Ser Leu Ala Asn Glu Ser Ala Ile Ser
 50 55 60

50

Pro Asp Val Pro Ala Gly Cys Arg Val Thr Phe Ala Gln Val Leu Ser
 65 70 75 80

Arg His Gly Ala Arg Tyr Pro Thr Glu Ser Lys Gly Lys Lys Tyr Ser
 85 90 95

55

EP 0 655 890 B1

	Ala	Leu	Ile	Glu	Glu	Ile	Gln	Gln	Asn	Val	Thr	Thr	Phe	Asp	Gly	Lys	
				100					105					110			
5	Tyr	Ala	Phe	Leu	Lys	Thr	Tyr	Asn	Tyr	Ser	Leu	Gly	Ala	Asp	Asp	Leu	
			115					120					125				
	Thr	Pro	Phe	Gly	Glu	Gln	Glu	Lys	Val	Asn	Ser	Gly	Ile	Lys	Phe	Tyr	
10		130					135					140					
	Gln	Arg	Tyr	Glu	Ser	Leu	Thr	Arg	Asn	Ile	Ile	Pro	Phe	Ile	Arg	Ser	
	145					150					155					160	
	Ser	Gly	Ser	Ser	Arg	Val	Ile	Ala	Ser	Gly	Glu	Lys	Phe	Ile	Glu	Gly	
15					165					170					175		
	Phe	Gln	Ser	Thr	Lys	Leu	Lys	Asp	Pro	Arg	Ala	Gln	Pro	Gly	Gln	Ser	
				180					185					190			
20	Ser	Pro	Lys	Ile	Asp	Val	Val	Ile	Ser	Glu	Ala	Ser	Ser	Ser	Asn	Asn	
			195					200						205			
	Thr	Leu	Asp	Pro	Gly	Thr	Cys	Thr	Val	Phe	Glu	Asp	Ser	Glu	Lys	Ala	
		210					215					220					
25	Asp	Thr	Val	Glu	Ala	Asn	Phe	Thr	Ala	Thr	Phe	Ala	Pro	Ser	Ile	Arg	
	225					230					235					240	
	Gln	Arg	Leu	Glu	Asn	Asp	Lys	Ser	Glu	Val	Thr	Leu	Thr	Asp	Thr	Glu	
30					245					250					255		
	Val	Thr	Tyr	Leu	Met	Asp	Met	Cys	Ser	Phe	Asp	Thr	Ile	Ser	Thr	Ser	
				260				265						270			
	Thr	Val	Asp	Thr	Lys	Leu	Ser	Pro	Phe	Cys	Asp	Leu	Phe	Thr	His	Asp	
35			275					280					285				
	Glu	Trp	Ile	His	Tyr	Asp	Tyr	Leu	Gln	Ser	Leu	Lys	Lys	Tyr	Tyr	Gly	
		290					295					300					
40	His	Gly	Ala	Gly	Asn	Pro	Leu	Gly	Pro	Thr	Gln	Gly	Val	Gly	Tyr	Ala	
	305					310					315					320	
	Asn	Glu	Leu	Ile	Ala	Arg	Leu	Thr	His	Ser	Pro	Val	His	Asp	Asp	Thr	
					325					330					335		
45	Ser	Ser	Asn	His	Thr	Leu	Asp	Ser	Asn	Pro	Ala	Thr	Phe	Pro	Leu	Asn	
				340					345					350			
	Ser	Thr	Leu	Tyr	Ala	Asp	Phe	Ser	His	Asp	Asn	Gly	Ile	Ile	Ser	Ile	
50			355					360					365				
	Leu	Phe	Ala	Leu	Gly	Leu	Tyr	Asn	Gly	Thr	Lys	Pro	Leu	Ser	Thr	Thr	
		370					375					380					
55	Thr	Val	Glu	Asn	Ile	Thr	Gln	Thr	Asp	Gly	Phe	Ser	Ser	Ala	Trp	Thr	
	385					390					395					400	

Val Pro Phe Ala Ser Arg Leu Tyr Val Glu Met Met Gln Cys Gln Ala
405 410 415

5 Glu Gln Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Val Pro
420 425 430

Leu His Gly Cys Pro Ile Asp Ala Leu Gly Arg Cys Thr Arg Asp Ser
435 440 445

10 Phe Val Arg Gly Leu Ser Phe Ala Arg Ser Gly Gly Asp Trp Ala Glu
450 455 460

Cys Ser Ala
465

15

(21) INFORMATION FOR SEQ ID NO:20:

[0196]

20

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2071 bases

(B) TYPE: nucleic acid

25 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

30 (ix) FEATURE:

OTHER INFORMATION: Sequence of Figure 11

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

35

GCATGCTGGA	CCGCAATCTC	CGATCGCCGG	GTATAAAAGG	TCCTCCAAAC	CCCTCTCGGT	60
CGATATGTAC	CCCGCTCGTC	ATCTCCAATC	CTCTCGAGAG	CACCTTCTCC	AGCTTTTGTC	120
AATTGTACCT	TCGCAATGCC	TCGCACCTCT	CTCCTCACCC	TGGCCTGTGC	TCTGGCCACG	180
GGCGCATCCG	CTTTCTCCTA	CGGCGCTGCC	ATTCTCAGT	CAACCCAGGA	GAAGCAGTTC	240
TCTCAGGAGT	TCCGCGATGG	CTACAGCATC	CTCAAGCACT	ACGGTGGTAA	CGGACCCTAC	300
TCCGAGCGTG	TGTCCTACGG	TATCGCTCGC	GATCCCCCGA	CCAGCTGCGA	GGTCGATCAG	360
GTCATCATGG	TCAAGCGTCA	CGGAGAGCGC	TACCCGTCCC	CTTCAGCCGG	CAAGGACATC	420
GAAGAGGCC	TGGCCAAGGT	CTACAGCATC	AACACTACTG	AATACAAGGG	CGACCTGGCC	480
TTCTTGAACG	ACTGGACCTA	CTACGTCCCT	AATGAGTGCT	ACTACAACGC	CGAGACCACC	540
AGCGGCCCT	ACGCCGGTTT	GCTGGACGCG	TACAACCATG	GCAACGATTA	CAAGGCTCGC	600
TACGGCCACC	TCTGGAACGG	TGAGACGGTC	GTGCCCTTCT	TTTCTAGTGG	CTACGGACGT	660
GTCATCGAGA	CGGCCCGCAA	GTTCGGTGAG	GGTTTCTTTG	GCTACAACCTA	CTCCACCAAC	720
GCTGCCCTCA	ACATCATCTC	CGAGTCCGAG	GTATGGGCG	CGGACAGCCT	CACGCCCACC	780
TGTGACACCG	ACAACGACCA	GACCACCTGC	GACAACCTGA	CTTACCAGCT	GCCCCAGTTC	840
AAGGTCGCTG	CTGCCCCGCT	AAACTCCCAG	AACCCCGGCA	TGAACCTCAC	CGCATCTGAT	900
GTCTACAACC	TGATGGGTAT	GTGATTACGG	TACAATCATT	GGCTCAAACC	TCCAGCTGAC	960
AGCATCCTAG	TTATGGCCTC	CTTTGAGCTC	AATGCTCGTC	CCTTCTCCAA	CTGGATCAAC	1020
GCCTTTACCC	AGGACGAATG	GGTCAGCTTC	GGTTACGTTG	AGGATTTGAA	CTACTACTAC	1080
TGCGCTGGGT	GAGTTTACCA	TTTGATCCAT	TATTGTCTTG	GATCAGCTAA	CGATCGATAG	1140
TCCCGGTGAC	AAGAACATGG	CTGCTGTGGG	TGCCGTCTAC	GCCAACGCCA	GTCTCACCTT	1200
CCTGAACCAG	GGACCCAAGG	AAGCCGGCTC	CTTGTTCTTC	AACTTGTACG	TTCTCGGCAG	1260
AATCAGAGTC	TCACAAAAAG	AAACTCTTCA	CTAACATATA	GTAGTGCCCA	CGACACCAAC	1320
ATCACCCCCA	TCCTCGCCGC	CCTAGGCGTC	CTCATCCCCA	ACGAGGACCT	TCCTCTTGAC	1380
CGGGTCGCCT	TCGGCAACCC	CTACTCGATC	GGCAACATCG	TGCCCATGGG	TGGCCATCTG	1440
ACCATCGAGC	GTCTCAGCTG	CCAGGCCACC	GCCCTCTCGG	ACGAGGGTAC	CTACGTGCGT	1500

```

5   CTGGTGCTGA ACGAGGCTGT ACTCCCCTTC AACGACTGCA CCTCCGGACC GGGCTACTCC 1560
    TGCCCTCTGG CCAACTACAC CTCCATCCTG AACAAAGAATC TGCCAGACTA CACGACCACC 1620
    TGCAATGTCT CTGCGTCCTA CCCGCGAGTAT CTGAGCTTCT GGTGGAAC TAACACCACG 1680
    ACGGAGCTGA ACTACCGCTC TAGCCCTATT GCCTGCCAGG AGGGTGATGC TATGGACTAG 1740
    ATGCAGAGGG GTAGGTCCCG GGATACTTTA GTGATGATTG ATATTCAAGT TTGGTGGTGA 1800
    CGATCACCTT GTTAATAGTC TTGTACAGTC ATACGGTGAA TGTAATAAAT GATAATAGCA 1860
    ATGATACATG TTGGAATCTC GTTTTGTCTT TTGTGTGCAT AGGCGCTTTG GGGGTGTATT 1920
    TTTAGGCGTT AGACTTATTT TCAATTCGTG TATAATGCGG TCAGTAAATG AATCATCAAT 1980
    TATTCAAATG CAATGCTGTA TACGTGAAAC TATTGGGTTA AGACGCAGCT ACTAGCTGAC 2040
10  TGCTTGTTTA CTTTCTGTGT ACACCGCATG C 2071

```

(22) INFORMATION FOR SEQ ID NO:21:

[0197]

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 479 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

OTHER INFORMATION: Protein encoded by SEQ ID NO:20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:-

```

Met  Pro  Arg  Thr  Ser  Leu  Leu  Thr  Leu  Ala  Cys  Ala  Leu  Ala  Thr  Gly
1      5      10      15
Ala  Ser  Ala  Phe  Ser  Tyr  Gly  Ala  Ala  Ile  Pro  Gln  Ser  Thr  Gln  Glu
20     25     30
Lys  Gln  Phe  Ser  Gln  Glu  Phe  Arg  Asp  Gly  Tyr  Ser  Ile  Leu  Lys  His
35     40     45
Tyr  Gly  Gly  Asn  Gly  Pro  Tyr  Ser  Glu  Arg  Val  Ser  Tyr  Gly  Ile  Ala
50     55     60
Arg  Asp  Pro  Pro  Thr  Ser  Cys  Glu  Val  Asp  Gln  Val  Ile  Met  Val  Lys
65     70     75     80
Arg  His  Gly  Glu  Arg  Tyr  Pro  Ser  Pro  Ser  Ala  Gly  Lys  Asp  Ile  Glu
85     90     95
Glu  Ala  Leu  Ala  Lys  Val  Tyr  Ser  Ile  Asn  Thr  Thr  Glu  Tyr  Lys  Gly
100    105    110
Asp  Leu  Ala  Phe  Leu  Asn  Asp  Trp  Thr  Tyr  Tyr  Val  Pro  Asn  Glu  Cys
115    120    125
Tyr  Tyr  Asn  Ala  Glu  Thr  Thr  Ser  Gly  Pro  Tyr  Ala  Gly  Leu  Leu  Asp
130    135    140
Ala  Tyr  Asn  His  Gly  Asn  Asp  Tyr  Lys  Ala  Arg  Tyr  Gly  His  Leu  Trp
145    150    155    160

```

EP 0 655 890 B1

	Asn	Gly	Glu	Thr	Val	Val	Pro	Phe	Phe	Ser	Ser	Gly	Tyr	Gly	Arg	Val	
					165					170					175		
5	Ile	Glu	Thr	Ala	Arg	Lys	Phe	Gly	Glu	Gly	Phe	Phe	Gly	Tyr	Asn	Tyr	
				180					185					190			
	Ser	Thr	Asn	Ala	Ala	Leu	Asn	Ile	Ile	Ser	Glu	Ser	Glu	Val	Met	Gly	
10			195					200					205				
	Ala	Asp	Ser	Leu	Thr	Pro	Thr	Cys	Asp	Thr	Asp	Asn	Asp	Gln	Thr	Thr	
		210					215					220					
	Cys	Asp	Asn	Leu	Thr	Tyr	Gln	Leu	Pro	Gln	Phe	Lys	Val	Ala	Ala	Ala	
15	225					230					235					240	
	Arg	Leu	Asn	Ser	Gln	Asn	Pro	Gly	Met	Asn	Leu	Thr	Ala	Ser	Asp	Val	
					245					250					255		
20	Tyr	Asn	Leu	Met	Val	Met	Ala	Ser	Phe	Glu	Leu	Asn	Ala	Arg	Pro	Phe	
				260					265					270			
	Ser	Asn	Trp	Ile	Asn	Ala	Phe	Thr	Gln	Asp	Glu	Trp	Val	Ser	Phe	Gly	
			275					280					285				
25	Tyr	Val	Glu	Asp	Leu	Asn	Tyr	Tyr	Tyr	Cys	Ala	Gly	Pro	Gly	Asp	Lys	
		290					295					300					
	Asn	Met	Ala	Ala	Val	Gly	Ala	Val	Tyr	Ala	Asn	Ala	Ser	Leu	Thr	Leu	
30	305					310					315					320	
	Leu	Asn	Gln	Gly	Pro	Lys	Glu	Ala	Gly	Ser	Leu	Phe	Phe	Asn	Phe	Ala	
					325					330					335		
	His	Asp	Thr	Asn	Ile	Thr	Pro	Ile	Leu	Ala	Ala	Leu	Gly	Val	Leu	Ile	
35				340					345					350			
	Pro	Asn	Glu	Asp	Leu	Pro	Leu	Asp	Arg	Val	Ala	Phe	Gly	Asn	Pro	Tyr	
			355					360					365				
40	Ser	Ile	Gly	Asn	Ile	Val	Pro	Met	Gly	Gly	His	Leu	Thr	Ile	Glu	Arg	
		370					375					380					
	Leu	Ser	Cys	Gln	Ala	Thr	Ala	Leu	Ser	Asp	Glu	Gly	Thr	Tyr	Val	Arg	
	385					390					395					400	
45	Leu	Val	Leu	Asn	Glu	Ala	Val	Leu	Pro	Phe	Asn	Asp	Cys	Thr	Ser	Gly	
				405						410					415		
	Pro	Gly	Tyr	Ser	Cys	Pro	Leu	Ala	Asn	Tyr	Thr	Ser	Ile	Leu	Asn	Lys	
				420					425					430			
50	Asn	Leu	Pro	Asp	Tyr	Thr	Thr	Thr	Cys	Asn	Val	Ser	Ala	Ser	Tyr	Pro	
			435					440					445				
	Gln	Tyr	Leu	Ser	Phe	Trp	Trp	Asn	Tyr	Asn	Thr	Thr	Thr	Glu	Leu	Asn	
55		450					455					460					

Tyr Arg Ser Ser Pro Ile Ala Cys Gln Glu Gly Asp Ala Met Asp
465 470 475

5

(23) INFORMATION FOR SEQ ID NO:22:

[0198]

10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

15

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(ix) FEATURE:

20

OTHER INFORMATION: Peptide #1081/N-phy

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

25

Leu Ala Val Pro Ala Ser Arg Asn Gln Ser Thr Xaa Asp Thr
5 10

(24) INFORMATION FOR SEQ ID NO: 23:

30

[0199]

(i) SEQUENCE CHARACTERISTICS:

35

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: Peptide

(ix) FEATURE:

OTHER INFORMATION: Peptide from A. ficuum phytase

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

50

Leu Ala Val Pro Ala Ser Arg Asn Gln Ser Ser Gly Asp Thr
5 10

(25) INFORMATION FOR SEQ ID NO:24:

55

[0200]

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(ix) FEATURE:

OTHER INFORMATION: Peptide #420/10 phy

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Leu Tyr Val Glu Met Met Gln Asn Gln Ala Glu Gln Thr Pro Leu Val
5 10 15

(26) INFORMATION FOR SEQ ID NO:25:

[0201]

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(ix) FEATURE:

OTHER INFORMATION: Peptide from *A. ficuum* phytase

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Met Met Gln Cys Gln Ala Glu Gln Glu Pro Leu Val Arg Val Leu Val
5 10 15

Asn Asp Arg Xaa
20

(27) INFORMATION FOR SEQ ID NO:26:

[0202]

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(ix) FEATURE

OTHER INFORMATION: Peptide #675

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Lys Asp Pro Arg

5

(28) INFORMATION FOR SEQ ID NO:27:

[0203]

10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

15 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

20

(ix) FEATURE

OTHER INFORMATION: Peptide from phosphatases (Ullah, 1991)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

25

Lys Asp Pro Arg Ala
5

30

(29) INFORMATION FOR SEQ ID NO:28:

[0204]

(i) SEQUENCE CHARACTERISTICS:

35

(A) LENGTH: 23 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

45

Phe Ser Tyr Gly Ala Ala Ile Pro Gln Ser Thr Gln Glu Lys Gln Phe
5 10 15

Ser Gln Glu Phe Arg Asp Gly
20

50

(30) INFORMATION FOR SEQ ID NO:29:

[0205]

55

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

10 Arg His Gly Xaa Arg Xaa Pro
5

(31) INFORMATION FOR SEQ ID NO:30:

15 **[0206]**

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 28 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: DNA

(ix) FEATURE:

OTHER INFORMATION: Primer UPPHOS

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GAATTCCGAG TCCGAGGTCA TGGGCGCG

28

35

(32) INFORMATION FOR SEQ ID NO:31:

[0207]

40 (i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 27 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

50

OTHER INFORMATION: Primer DOWNPHOS

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

55

GAATTCCCGG GACCTACCCC TCTGCAT

27

(33) INFORMATION FOR SEQ ID NO:32:

[0208]

5 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

15 OTHER INFORMATION: Primer AP273

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

20 CTACCCCTCT GCATCTAG

18

(34) INFORMATION FOR SEQ ID NO:33:

[0209]

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 30 bases
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA

(ix) FEATURE:

OTHER INFORMATION: Oligo PHY-40

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

AGAAGAAATT TCTAGAACAG CAGCGATTGG

30

(35) INFORMATION FOR SEQ ID NO:34:

[0210]

50 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 bases
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 55 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

OTHER INFORMATION: Oligo #271

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CGAGAGCACC TTCTCTAGAT TTTGTCAAAT GTACC

35

10

(36) INFORMATION FOR SEQ ID NO:35:

[0211]

15 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

20 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

25

OTHER INFORMATION: Oligo #272

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

30

ACCCTCACCG AACTTGCGGG CCG

23

(37) INFORMATION FOR SEQ ID NO:36:

35

[0212]

(i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 18 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: Peptide

(ix) FEATURE:

OTHER INFORMATION: Peptide from A. niger phytase

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Ala Gln Val Leu Ser Arg His Gly Ala Arg Tyr Pro Thr Glu Ser Lys
5 10 15

55

Gly Lys

(38) INFORMATION FOR SEQ ID NO:37:

[0213]

5 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

(ix) FEATURE:

15 OTHER INFORMATION: Peptide from A. niger pH2.5 AP

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

20 Val Ile Met Val Lys Arg His Gly Glu Arg Tyr Pro Ser Pro Ser Ala
 5 10 15
 25 Gly Lys

(39) INFORMATION FOR SEQ ID NO:38:

[0214]

30 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 amino acids
 (B) TYPE: amino acid
 35 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

40 (ix) FEATURE:

OTHER INFORMATION: Peptide from E. coli APPA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

45 Val Val Ile Val Ser Arg His Gly Val Arg Ala Pro Thr Lys Ala Thr
 5 10 15
 50 Gln Leu

(40) INFORMATION FOR SEQ ID NO:39:

[0215]

55 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: Peptide

(ix) FEATURE:

OTHER INFORMATION: Peptide from yeast PHO-1

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

15 Val His Thr Leu Gln Arg His Gly Ser Arg Asn Pro Thr Gly Gly Asn
5 10 15
Ala Ala

20 (41) INFORMATION FOR SEQ ID NO:40:

[0216]

(i) SEQUENCE CHARACTERISTICS:

25

(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: Peptide

(ix) FEATURE:

OTHER INFORMATION: Peptide from yeast PHO-5

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

40 Leu Gln Met Val Gly Arg His Gly Glu Arg Tyr Pro Thr Val Ser Leu
5 10 15
Ala Lys

45 (42) INFORMATION FOR SEQ ID NO:41:

[0217]

(i) SEQUENCE CHARACTERISTICS:

50

(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

55

(ii) MOLECULE TYPE: Peptide

(ix) FEATURE:

OTHER INFORMATION: Peptide from yeast PHO-3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

5 Leu Gln Met Leu Ala Arg His Gly Glu Arg Tyr Pro Thr Tyr Ser Lys
5 10 15
10 Gly Ala

(43) INFORMATION FOR SEQ ID NO:42:

[0218]

15 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
20 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

25 (ix) FEATURE:

OTHER INFORMATION: Peptide from rat LAP

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

30 Val Thr Leu Leu Tyr Arg His Gly Asp Arg Ser Pro Val Lys Ala Tyr
5 10 15
35 Pro Lys

(44) INFORMATION FOR SEQ ID NO:43:

40 **[0219]**

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 amino acids
45 (B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Peptide

50 (ix) FEATURE:

OTHER INFORMATION: Peptide from rat PAP

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

EP 0 655 890 B1

Val Thr Leu Val Phe Arg His Gly Asp Arg Gly Pro Ile Glu Thr Phe
5 10 15

5 Pro Asn

(45) INFORMATION FOR SEQ ID NO:44:

10 [0220]

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: Peptide

(ix) FEATURE:

OTHER INFORMATION: Peptide from human ACP2

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Val Thr Leu Leu Tyr Arg His Gly Asp Arg Ser Pro Val Lys Thr Tyr
5 10 15

30 Pro Lys

(46) INFORMATION FOR SEQ ID NO:45:

35 [0221]

(i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: Peptide

(ix) FEATURE:

OTHER INFORMATION: Peptide from human ACPP

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

Val Thr Leu Val Phe Arg His Gly Asp Arg Ser Pro Ile Asp Thr Phe
5 10 15

55 Pro Thr

(47) INFORMATION FOR SEQ ID NO:46:

[0222]

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 84 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

OTHER INFORMATION: Oligo 260

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

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CTAGACACCT CAGCAATGTC GTTCCGATCT CTACTCGCCC TGAGCGGCCT CGTCTGCACA 60
GGGTTGGCAC TGGCAGTCCC CGCC 84

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(48) INFORMATION FOR SEQ ID NO:47:

[0223]

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 84 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

OTHER INFORMATION: Oligo 261

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

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TCGAGGCGGG GACTGCCAGT GCCAACCCTG TGCAGACGAG GCCGCTCAGG GCGAGTAGAG 60
ATCGGAACGA CATTGCTGAG GTGT 84

```

Claims

1. A recombinant transformed host cell **characterized in that** it has been transformed by a first nucleic acid capable of hybridising under stringent conditions with the nucleotide sequence of SEQ ID NO. 18 and a second nucleic acid capable of hybridising under stringent conditions with the nucleotide sequence of SEQ ID NO. 20 and that it is capable of secreting the pH 2.5 acid phosphatase enzyme activity and the phytase enzyme activity in a ratio of 3:1 to 16:1.
2. The host cell of claim 1, **characterized in that** it is capable of synthesizing and secreting a recombinant phytase and one or more recombinant phosphatases.

3. The host cell of claim 2, **characterized in that** said secreted overexpressed phytase has an enzyme activity per milliliter that is at least about 2-fold greater than a phytase enzyme activity secreted by ALKO243 (ATCC#38854) or said secreted overexpressed phosphatase has an enzyme activity that is at least about 10-fold greater than a phosphatase enzyme activity secreted by ALKO243 (ATCC#38854).
4. The host cell of claim 1, **characterized in that** it is a cell of a filamentous fungus.
5. The host cell of claim 4, **characterized in that** it is a cell of *Aspergillus*.
6. The host cell of claim 1, **characterized in that** the second nucleotide sequence encodes a conserved polypeptide having an amino acid sequence RHGX RXP, wherein R is arginine, H is histidine, G is glycine, X is any amino acid and P is proline.
7. The host cell of claim 1 **characterized in that** it secretes a pH 2.5 acid phosphatase polypeptide having a molecular weight of about 52,000 daltons to about 53,000 daltons as calculated from the predicted amino acid sequence.
8. The host cell of claim 7, **characterized in that** the pH 2.5 acid phosphatase is a tetrameric enzyme under non-denaturing conditions with an isoelectric point of about 4.0 to about 4.25, and has monomer molecular weight under denaturing conditions of about 66,000 daltons as determined by SDS-PAGE and about 46,000 daltons to about 49,000 daltons after substantial removal of carbohydrate.
9. The host cell of claim 8, **characterized in that** the pH 2.5 acid phosphatase has an apparent K_m of about 0.7 mM for Na-phytate and 4 mM for paranitrophenyl phosphate and a pH optimum of about pH 2.0 to about pH 2.5 at 37°C.
10. The host cell of claim 1, **characterized in that** has been transformed by a vector construct for expressing a phytase nucleic acid in a cell, comprising a promoter, the nucleic acid capable of hybridising under stringent conditions with the nucleotide sequence of SEQ ID NO. 18, and optionally a selectable marker.
11. The host cell of claim 1, **characterized in that** it has been transformed with a vector construct for expressing pH 2.5 acid phosphatase nucleic acid in a cell comprising a promoter, the nucleic acid capable of hybridizing under stringent conditions with the nucleotide sequence of SEQ ID NO. 20 and optionally a selectable marker.
12. The host cell of claim 10 or 11, **characterized in that** the vector constructs have a promoter selected from among a β -tubulin, GA, GAPDH, native phytase and native pH 2.5 acid phosphatase promoter.
13. The host cell of claim 1, **characterized in that** it has been transformed with a vector construct for expressing phytase and phosphatase nucleic acids in a cell, comprising at least one promoter, the nucleotide sequences of SEQ ID NO. 18 and SEQ ID NO. 20 and optionally one or more selectable markers.
14. The host cell of claim 10, **characterized in that** the vector construct is selected from the group of vectors as shown in Figs. 10A, 10B, 10C, 10D, 12A, 12B, 12C and 12D, respectively.
15. The host cell of claim 11, **characterized in that** the vector construct is selected from the group of vectors as shown in Figs. 13A, 13B, 13C and 13D, respectively.
16. The host cell of claim 13, **characterized in that** the vector construct is selected from a group of vectors as shown in Figs. 14A and 14B, respectively.
17. Use of the host cells of one of claims 1 to 16 for producing an enzymatically active mixture comprising one or more overexpressed phytate degrading enzymes in a ratio of pH 2.5 acid phosphatase to phytase enzyme activity of 3:1 to 16:1.

Patentansprüche

1. Rekombinante transformierte Wirtszelle, **dadurch gekennzeichnet, dass** sie durch eine erste Nucleinsäure, die fähig ist, unter stringenten Bedingungen mit der Nucleotidsequenz SEQ ID NO. 18 zu hybridisieren, und eine zweite Nucleinsäure, die fähig ist, unter stringenten Bedingungen mit der Nucleotidsequenz SEQ ID NO. 20 zu

hybridisieren, transformiert worden ist, und dass sie fähig ist, die pH 2,5-saure Phosphatase-Enzymaktivität und die Phytase-Enzymaktivität in einem Verhältnis von 3:1 bis 16:1 zu sezernieren.

2. Wirtszelle nach Anspruch 1, **dadurch gekennzeichnet, dass** sie fähig ist, eine rekombinante Phytase und eine oder mehrere rekombinante Phosphatase(n) zu synthetisieren und zu sezernieren.
3. Wirtszelle nach Anspruch 2, **dadurch gekennzeichnet, dass** die sezernierte, überexprimierte Phytase eine Enzymaktivität pro ml hat, die zweimal höher ist als eine Phytase-Enzymaktivität, die durch ALK0243 (ATCC#38854) sezerniert wird, oder die sezernierte überexprimierte Phosphatase eine Enzymaktivität hat, die mindestens etwa 10 mal höher ist als eine Phosphatase-Enzymaktivität, die durch ALK0243 (ATCC#38854) sezerniert wird.
4. Wirtszelle nach Anspruch 1, **dadurch gekennzeichnet, dass** sie eine Zelle eines Fadenpilzes ist.
5. Wirtszelle nach Anspruch 4, **dadurch gekennzeichnet, dass** sie eine Zelle von *Aspergillus* ist.
6. Wirtszelle nach Anspruch 1, **dadurch gekennzeichnet, dass** die zweite Nucleotidsequenz für ein konserviertes Polypeptid codiert, das eine Aminosäuresequenz RHGXRX hat, worin R Arginin ist, H Histidin ist, G Glycin ist, X eine beliebige Aminosäure ist und P Prolin ist.
7. Wirtszelle nach Anspruch 1, **dadurch gekennzeichnet, dass** sie ein pH 2,5-saure Phosphatase-Polypeptid mit einem Molekulargewicht von etwa 52.000 Dalton bis etwa 53.000 Dalton, wie es aus der vorhergesagten Aminosäuresequenz errechnet wird, sezerniert.
8. Wirtszelle nach Anspruch 7, **dadurch gekennzeichnet, dass** die pH 2,5-saure Phosphatase ein tetrameres Enzym unter nicht denaturierenden Bedingungen mit einem isoelektrischen Punkt von etwa 4,0 bis etwa 4,25 ist und ein Monomeren-Molekulargewicht unter denaturierenden Bedingungen von etwa 66.000 Dalton, bestimmt durch SDS-Page und etwa 46.000 Dalton bis etwa 49.000 Dalton nach substantieller Kohlenhydratentfernung hat.
9. Wirtszelle nach Anspruch 8, **dadurch gekennzeichnet, dass** die pH 2,5-saure Phosphatase einen scheinbaren Km-Wert von etwa 0,7 mM für Na-Phytat und 4 mM für Paranitrophenylphosphat und ein pH-Optimum von etwa pH 2,0 bis etwa pH 2,5 bei 37°C hat.
10. Wirtszelle nach Anspruch 1, **dadurch gekennzeichnet, dass** sie durch ein Vektorkonstrukt zum Exprimieren einer Phytasenucleinsäure in einer Zelle transformiert worden ist, umfassend einen Promotor, die Nucleinsäure, die fähig ist, unter stringenten Bedingungen mit der Nucleotidsequenz SEQ ID NO. 18 zu hybridisieren, und gegebenenfalls einen selektierbaren Marker.
11. Wirtszelle nach Anspruch 1, **dadurch gekennzeichnet, dass** sie mit einem Vektorkonstrukt zum Exprimieren von pH 2,5-saure Phosphatase-Nucleinsäure transformiert worden ist, umfassend einen Promotor, die Nucleinsäure, die fähig ist, unter stringenten Bedingungen mit der Nucleotidsequenz SEQ ID NO. 20 zu hybridisieren, und gegebenenfalls einen selektierbaren Marker.
12. Wirtszelle nach Anspruch 10 oder 11, **dadurch gekennzeichnet, dass** die Vektorkonstrukte einen Promotor haben, der aus einem β -Tubulin-, GA-, GAPHD-, nativen Phytase- und nativen pH 2,5-saure Phosphatase-Promotor ausgewählt ist.
13. Wirtszelle nach Anspruch 1, **dadurch gekennzeichnet, dass** sie mit einem Vektorkonstrukt zum Exprimieren von Phytase und Phosphatase-Nucleinsäuren in einer Zelle transformiert worden ist, umfassend mindestens einen Promotor, die Nucleotidsequenzen SEQ ID NO. 18 und SEQ ID NO. 20 und gegebenenfalls einen selektierbaren Marker oder mehrere selektierbare Marker.
14. Wirtszelle nach Anspruch 10, **dadurch gekennzeichnet, dass** das Vektorkonstrukt aus der Gruppe, bestehend aus Vektoren wie sie in Figur 10A, 10B, 10C, 10D, 12A, 12B, 12C und 12D dargestellt sind, ausgewählt ist.
15. Wirtszelle nach Anspruch 11, **dadurch gekennzeichnet, dass** das Vektorkonstrukt aus der Gruppe von Vektoren, wie sie in Figuren 13A, 13B, 13C bzw. 13D dargestellt sind, ausgewählt ist.
16. Wirtszelle nach Anspruch 13, **dadurch gekennzeichnet, dass** das Vektorkonstrukt aus einer Gruppe von Vektoren

ren, wie sie in Figur 14A bzw. 14B dargestellt sind, ausgewählt ist.

17. Verwendung der Wirtszelle nach einem der Ansprüche 1 bis 16 zur Herstellung eines enzymatisch aktiven Gemisches, das ein überexprimiertes Phytat abbauendes Enzym oder mehrere überexprimierte Phytat abbauende Enzyme in einem Verhältnis von pH 2,5-saure Phosphatase- zu Phytase-Enzymaktivität von 3:1 bis 16:1 umfasst.

Revendications

1. Cellule hôte transformée recombinante **caractérisée en ce qu'elle** a été transformée par un premier acide nucléique capable de s'hybrider dans des conditions rigoureuses avec la séquence de nucléotides de SEQ ID NO. 18 et avec un deuxième acide nucléique capable de s'hybrider dans des conditions rigoureuses avec la séquence de nucléotides de SEQ ID n° 20 et **en ce qu'elle** est capable de sécréter l'activité enzymatique d'une phosphatase acide active à pH 2,5 et l'activité enzymatique d'une phytase dans un rapport de 3:1 à 16:1.
2. Cellule hôte selon la revendication 1, **caractérisée en ce qu'elle** est capable de synthétiser et de sécréter une phytase recombinante et une ou plusieurs phosphatases recombinantes.
3. Cellule hôte selon la revendication 2, **caractérisée en ce que** ladite phytase surexprimée sécrétée a une activité enzymatique par millilitre qui est au moins environ deux fois supérieure à l'activité enzymatique d'une phytase sécrétée par ALK0243 (ATCC#38854) ou **en ce que** ladite phosphatase surexprimée sécrétée a une activité enzymatique qui est au moins environ 10 fois supérieure à l'activité enzymatique d'une phosphatase sécrétée par ALK0243 (ATCC#38854).
4. Cellule hôte selon la revendication 1, **caractérisée en ce qu'il** s'agit d'une cellule d'un champignon filamenteux.
5. Cellule hôte selon la revendication 4, **caractérisée en ce qu'il** s'agit d'une cellule d'*Aspergillus*.
6. Cellule hôte selon la revendication 1, **caractérisée en ce que** la deuxième séquence de nucléotides code pour un polypeptide conservé ayant une séquence d'acides aminés RHGXRX, dans laquelle R représente l'arginine, H représente l'histidine, G représente la glycine, X représente un acide aminé quelconque et P représente la proline.
7. Cellule hôte selon la revendication 1, **caractérisée en ce qu'elle** sécrète un polypeptide de phosphatase acide active à pH 2,5 ayant une masse moléculaire d'environ 52 000 daltons à environ 53 000 daltons telle que calculée d'après la séquence d'acides aminés prédite.
8. Cellule hôte selon la revendication 7, **caractérisée en ce que** la phosphatase acide active à pH 2,5 est une enzyme tétramère dans des conditions non dénaturantes ayant un point isoélectrique d'environ 4,0 à environ 4,25 et **en ce qu'elle** possède une masse moléculaire de monomère, dans des conditions dénaturantes, d'environ 66 000 daltons telle que déterminée par SDS-PAGE et d'environ 46 000 daltons à environ 49 000 daltons après une élimination importante des sucres.
9. Cellule hôte selon la revendication 8, **caractérisée en ce que** la phosphatase acide active à pH 2,5 possède un K_m apparent d'environ 0,7 mM pour le phytate sodique et de 4 mM pour le phosphate de paranitrophényle et un pH optimal dans la gamme de pH allant d'environ 2,0 à environ 2,5, à 37°C.
10. Cellule hôte selon la revendication 1, **caractérisée en ce qu'elle** a été transformée par un construct de vecteur pour exprimer un acide nucléique de phytase dans une cellule, comprenant un promoteur, l'acide nucléique capable de s'hybrider dans des conditions rigoureuses avec la séquence de nucléotides de SEQ ID n° 18 et éventuellement un marqueur sélectionnable.
11. Cellule hôte selon la revendication 1, **caractérisée en ce qu'elle** a été transformée par un vecteur élaboré pour exprimer un acide nucléique de phosphatase acide active à pH 2,5 dans une cellule, comprenant un promoteur, l'acide nucléique capable de s'hybrider dans des conditions rigoureuses avec la séquence de nucléotides de SEQ ID n° 20 et éventuellement un marqueur sélectionnable.
12. Cellule hôte selon la revendication 10 ou 11, **caractérisée en ce que** les constructs de vecteur possèdent un promoteur choisi parmi une β -tubuline, GA, GAPHD, un promoteur de phytase d'origine naturelle et un promoteur

de phosphatase acide active à pH 2,5 d'origine naturelle.

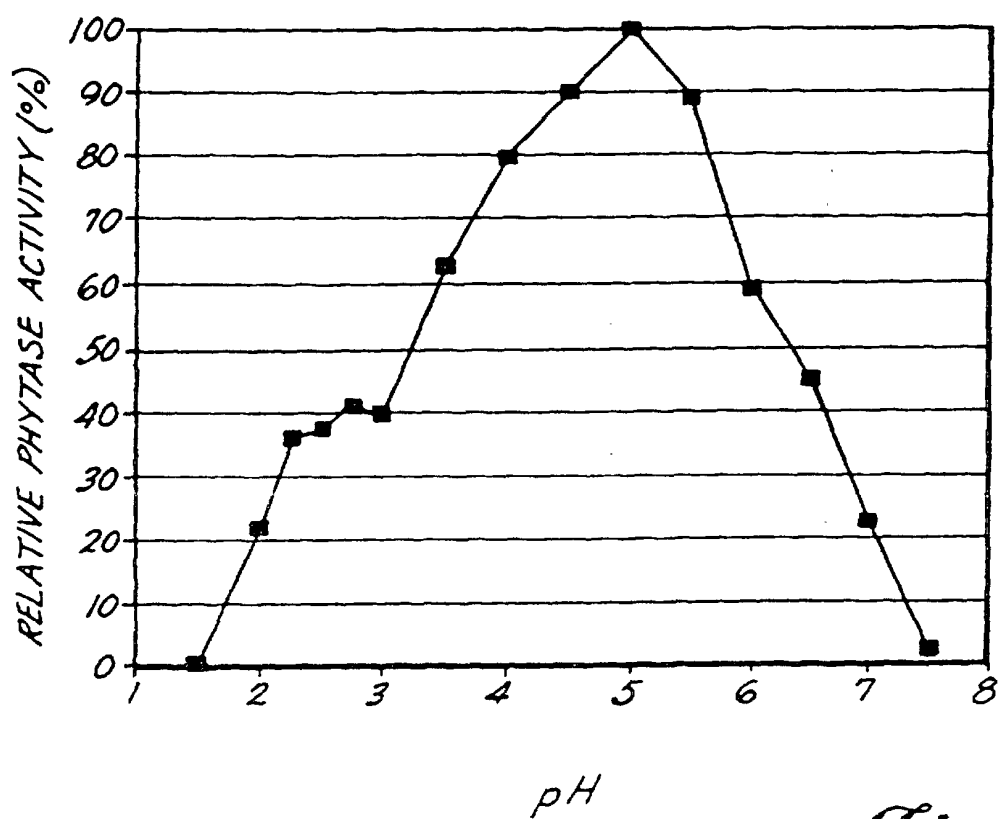
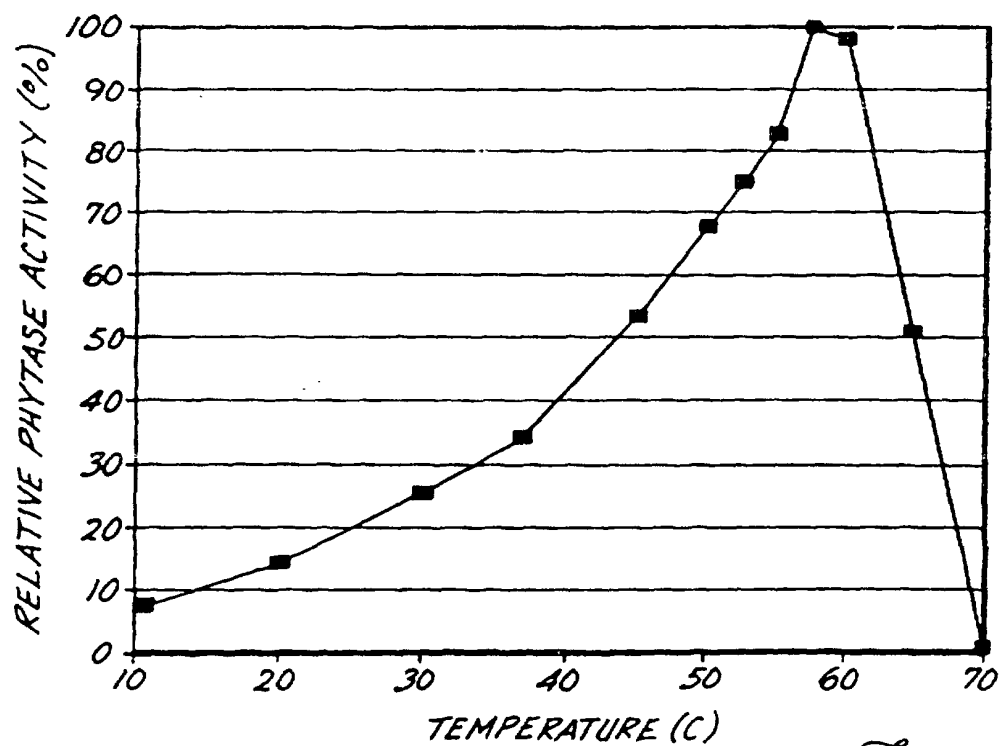
13. Cellule hôte selon la revendication 1, **caractérisée en ce qu'elle** a été transformée par un construct de vecteur pour exprimer des acides nucléiques de phytase et de phosphatase dans une cellule, comprenant au moins un promoteur, les séquences de nucléotides de SEQ ID n° 18 et de SEQ ID n° 20 et éventuellement un ou plusieurs marqueurs sélectionnables.

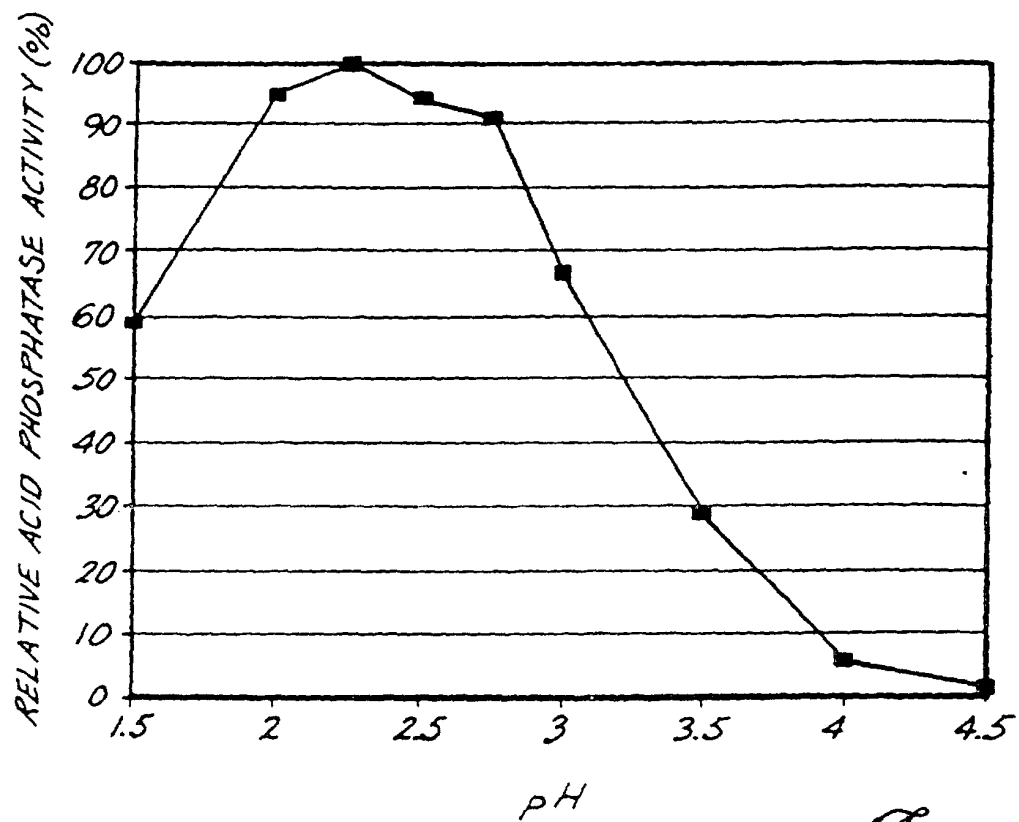
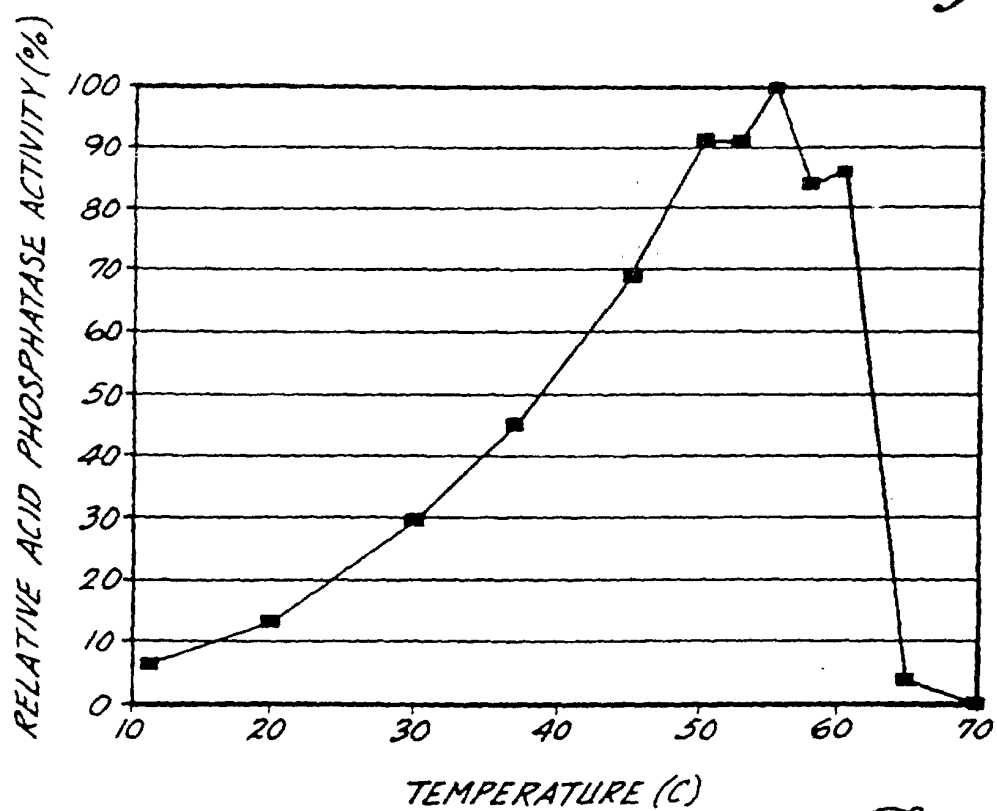
14. Cellule hôte selon la revendication 10, **caractérisée en ce que** le construct de vecteur est choisi dans le groupe de vecteurs représentés respectivement dans les figures 10A, 10B, 10C, 10D, 12A, 12B, 12C et 12D.

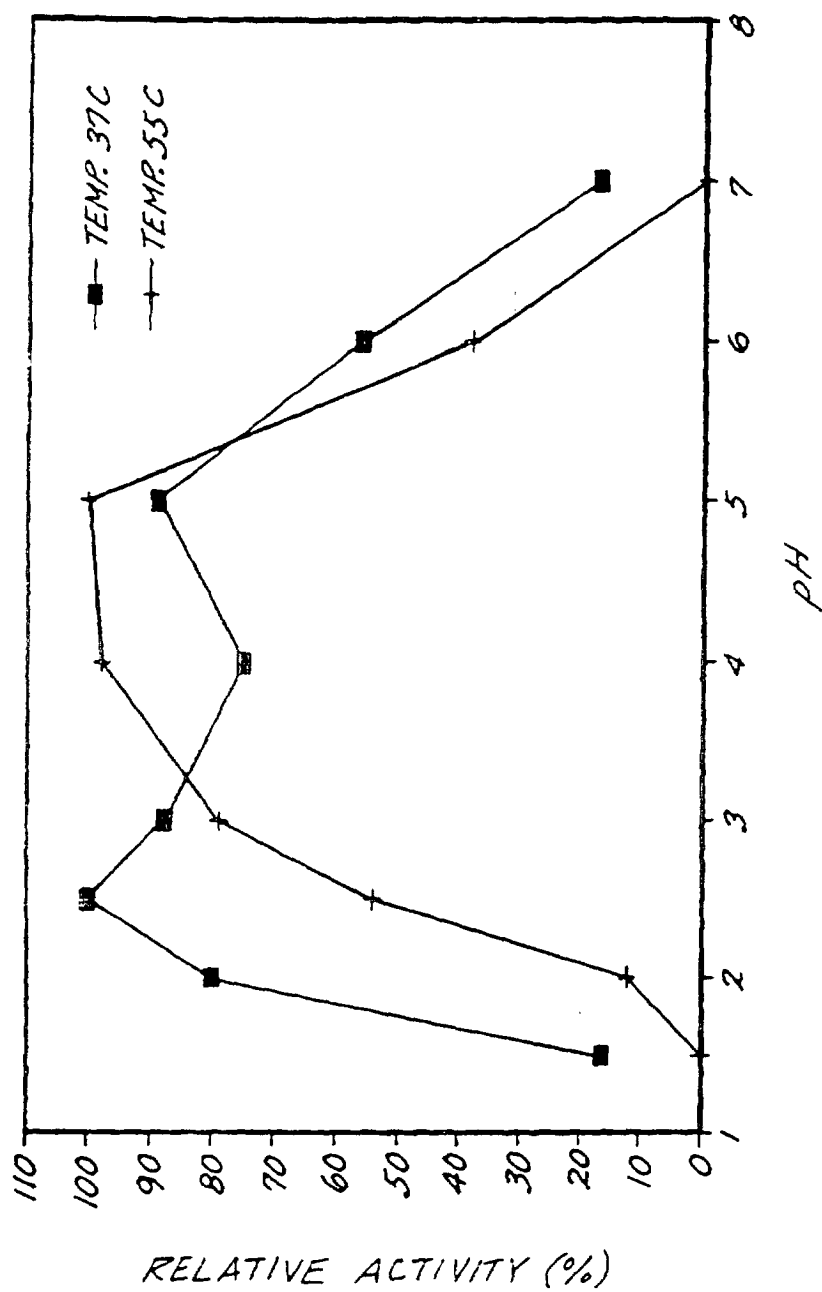
15. Cellule hôte selon la revendication 11, **caractérisée en ce que** le construct de vecteur est choisi dans le groupe de vecteurs représentés respectivement dans les figures 13A, 13B, 13C et 13D.

16. Cellule hôte selon la revendication 13, **caractérisée en ce que** le construct de vecteur est choisi dans le groupe de vecteurs représentés respectivement dans les figures 14A et 14B.

17. Utilisation des cellules hôtes selon les revendications 1 à 16 pour produire un mélange à activité enzymatique comprenant une ou plusieurs enzymes de dégradation du phytate surexprimées dans un rapport d'activité enzymatique de phosphatase acide active à pH 2,5 /phytase de 3:1 à 16:1.

*Fig. 1.**Fig. 2.*

*Fig. 3.**Fig. 4.*

*Fig. 5.*

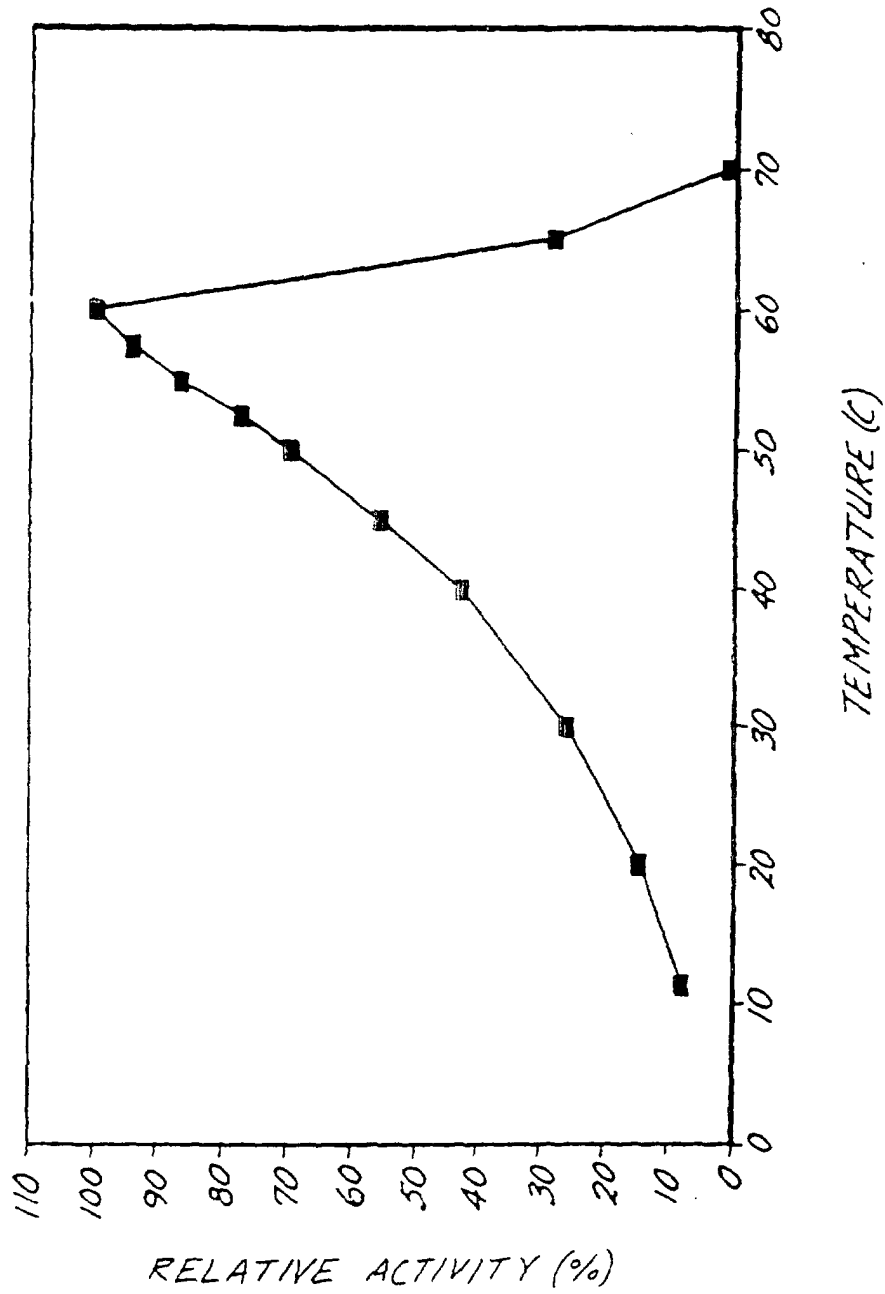


Fig. 6.

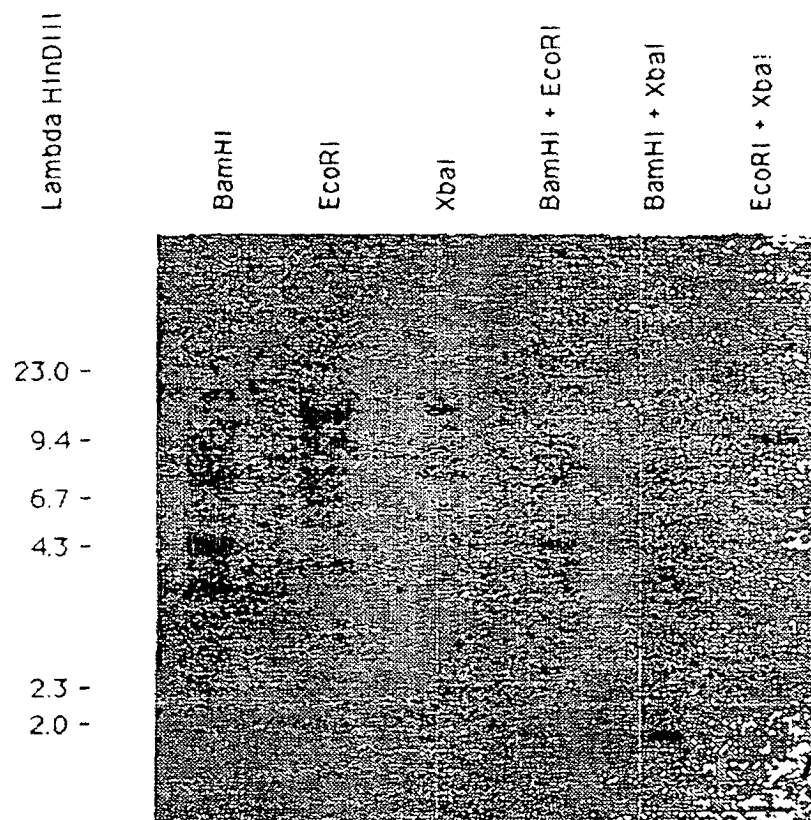


Fig. 7.

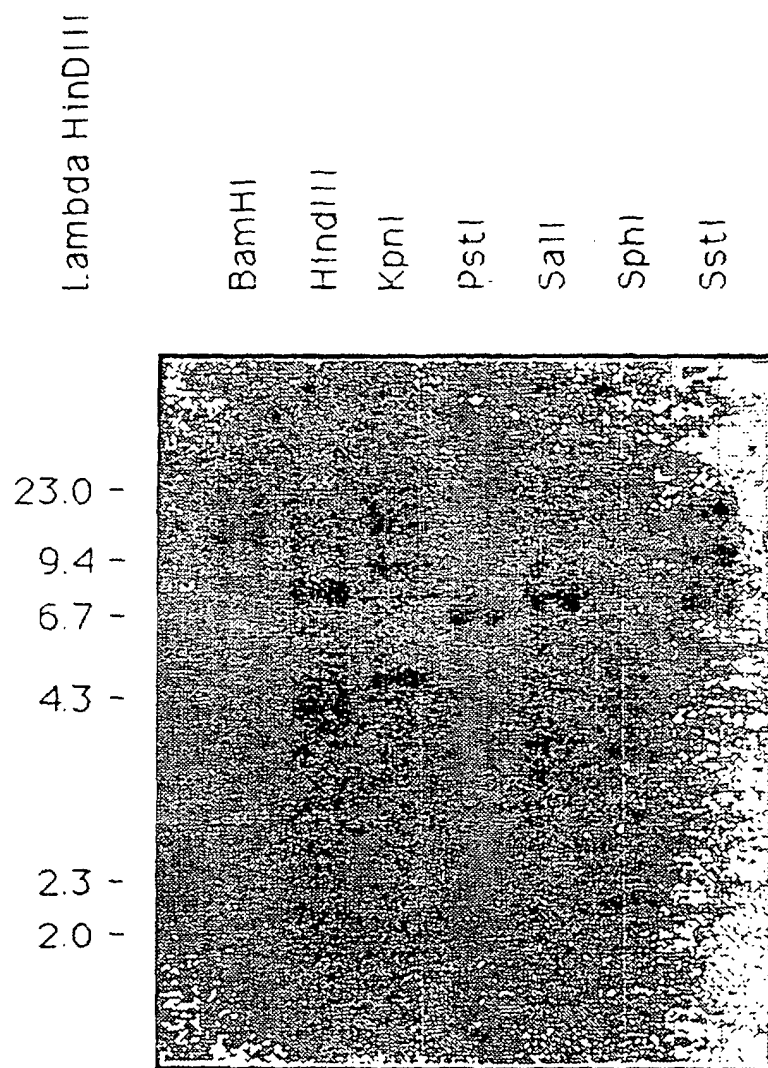


Fig. 8.

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1  TGATGGCGC CTAGGGCATC CAGGCACCTT TTCCCAACGG GGAACCTCC GTTGCCACG TGCCCTGGT CAGCAATCA AAGCGTCCCA CGGCAATGCT
101 GGATCAACGA TCAACTTCAA TGCAATAAAT GAAGATGCAA CTACACCAT CTGTGCTT TCCTCCGAGA AAGCTCCTCC ACTTCACACA CTAGATTTAT
201 CCGTTCCTTG TCGACTTCCC GTCCCATTCG GCGTCGTCCA CTGAAGATCT ATCCACCAT TGCACGIGGG CCACCTTGT GAGCTTCTAA CTGAACATGG
301 TAGAGTATCA CACAACATGC GAAAGTGGGA TGAAGGGGT ATATCAGGAC CGTCCGGTCC GCGCGATGG CCGTAGCTGC CAATCGCTGC TGTGCAAGAA
401 ATTCTTCTC ATAGGCATCA TGGCGCTC TCGTGTCTA CTTCCTTGT ATCTCCTAGC TGGTATGCT AAGCACCGCT ATCTAAGTCT GATAAGGACC
      M G V S A V L L P L Y L L A G>
501 CTCTTTGCCG AGGGCCCCCTG AAGCTCGGAC TGTGTGGGAC TACIGATCGC TACAAATCTG TGCAGAGTCA CTCTCGGACT GGCAGTCCCC GCCTCGAGAA
      V T S G L A V P A S R>
601 ATCAATCCAC TTGCGATACG GTCGATCAAG GGTATCAATG CTTCCTCAG ACTTCGCATC TTGGGGTCA ATACCGCGCG TTCTTCTCTC TGGCAACGA
      N Q S T C D T V D Q G Y Q C F S E T S H L W G Q Y A P F F S L A N E>
701 ATCGGCCATC TCCCTTGATG TCGCCGCGCG TTGCAGAGTCT ACTTTCGCTC AGGTCCCTC CCGTCATGGA GCGCGTATC CGACCGAGT CAGGGGCAAG
      S A I S P D V P A G C R V T F A Q V L S R H G A R Y P T E S K G K>
801 AATACTCCG CTCTCATTTGA GCGATCCAG CAGAACGTGA CCACCTTGA TGGAAATAT GCGTCTCTGA AGACATACAA CTACAGCTTG GGTGCGATG
      K Y S A L I E E I Q Q N V T T F D G K Y A F L K T Y N Y S L G A D>
901 ACCTACTCC CTTCGGAGAG CAGGAGCTAG TCAACTCCG CATCAAGTCT TACCAGGAT ACGAATCGCT CACAAGGAAC ATCATTCCTG TCACTCGATC
      D L T P F G E Q E L V N S G I K F Y Q R Y E S L T R N I I P F I R S>
      >BamH1
1001 CTCTGGCTCC AGCGCGTGA TCGCTCCGG CGAGAAATTC ATTGAGGCT TCCAGAGCAC CAAGCTGAAG GATCTCTGTG CCCAGCGGG CCAATCGTCTG
      S G S S R V I A S G E K F I E G F Q S T K L K D P R A Q P G Q S S>
1101 CCCAAGATCG ACGTGGTCAI TTCCGAGGCC AGCTCATCCA ACAACATCT CGACCCAGGC ACCTGCACTG TCTTTGAAGA CAGCGAATG GCCGATACCG
      P K I D V V I S E A S S S N N T L D P G T C T V F E D S E L A D T>
1201 TCGAAGCCAA TTTACCGCC ACGTTCGCC CCTCCATTCG TCAACGCTG GAGAACGACC TGTCTGGCT GACTCTCACA GACACAGAAG TGACCTACCT
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```

Fig. 9A.

Fig. 9B.

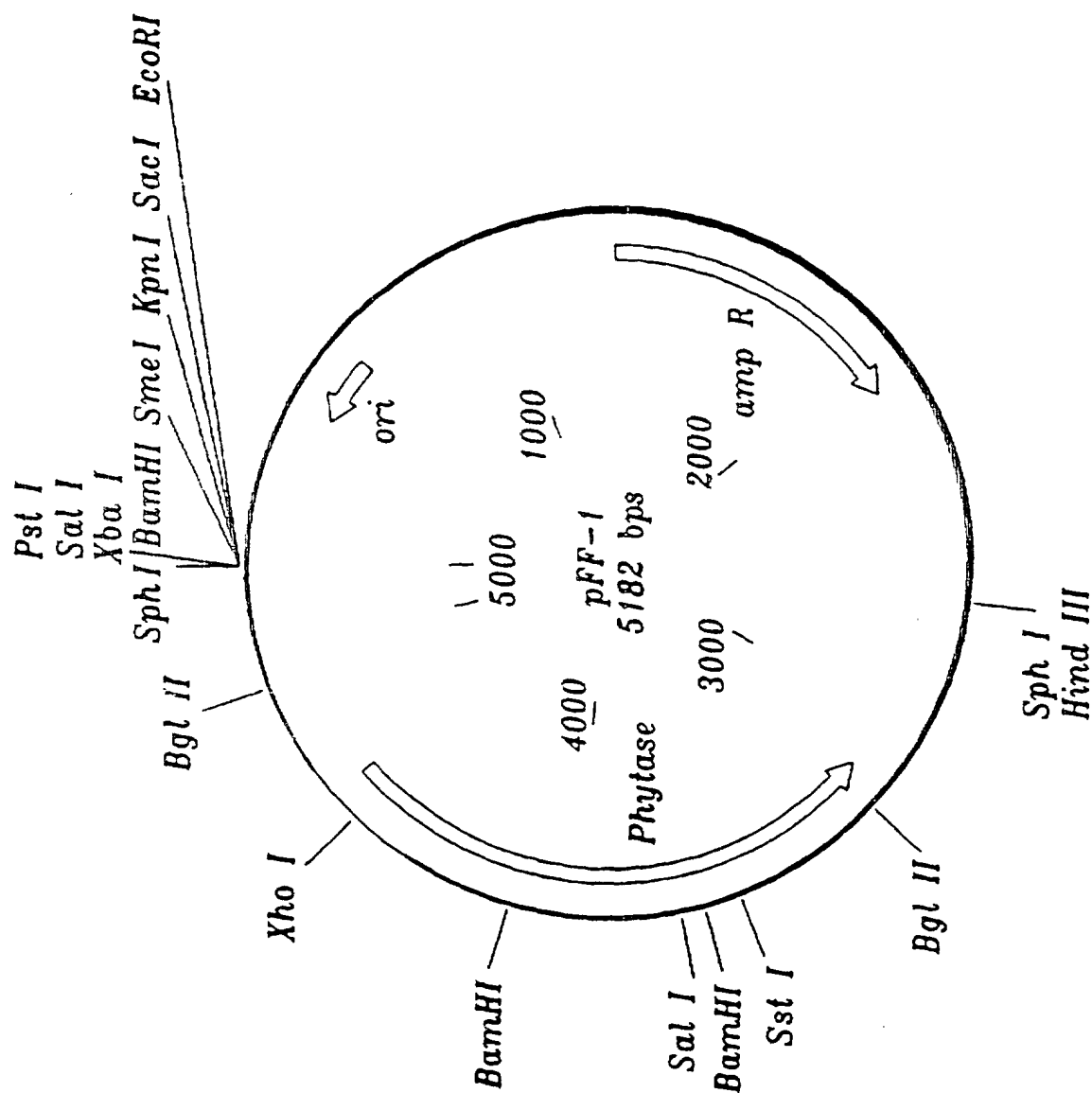


Fig. 10A.

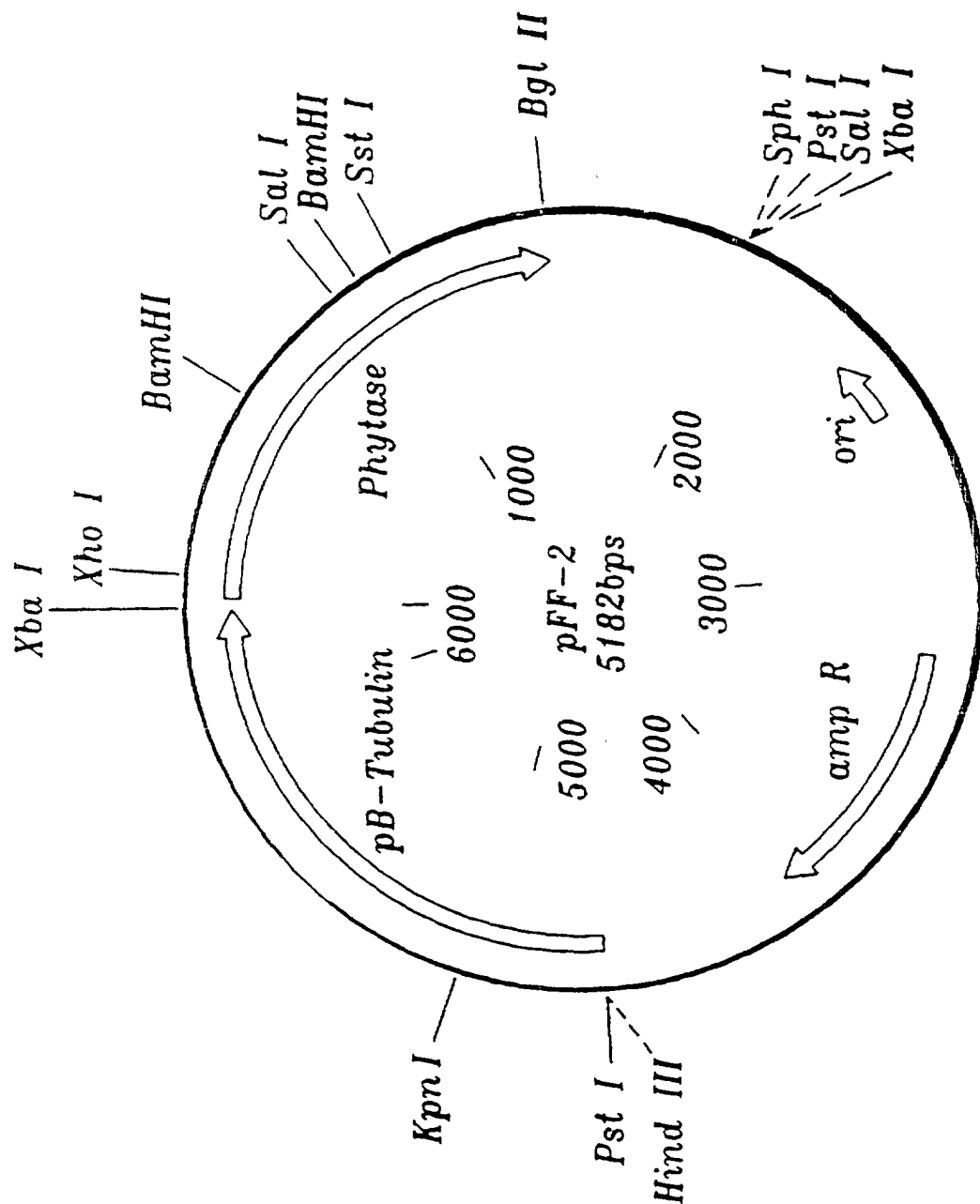


Fig. 10B.

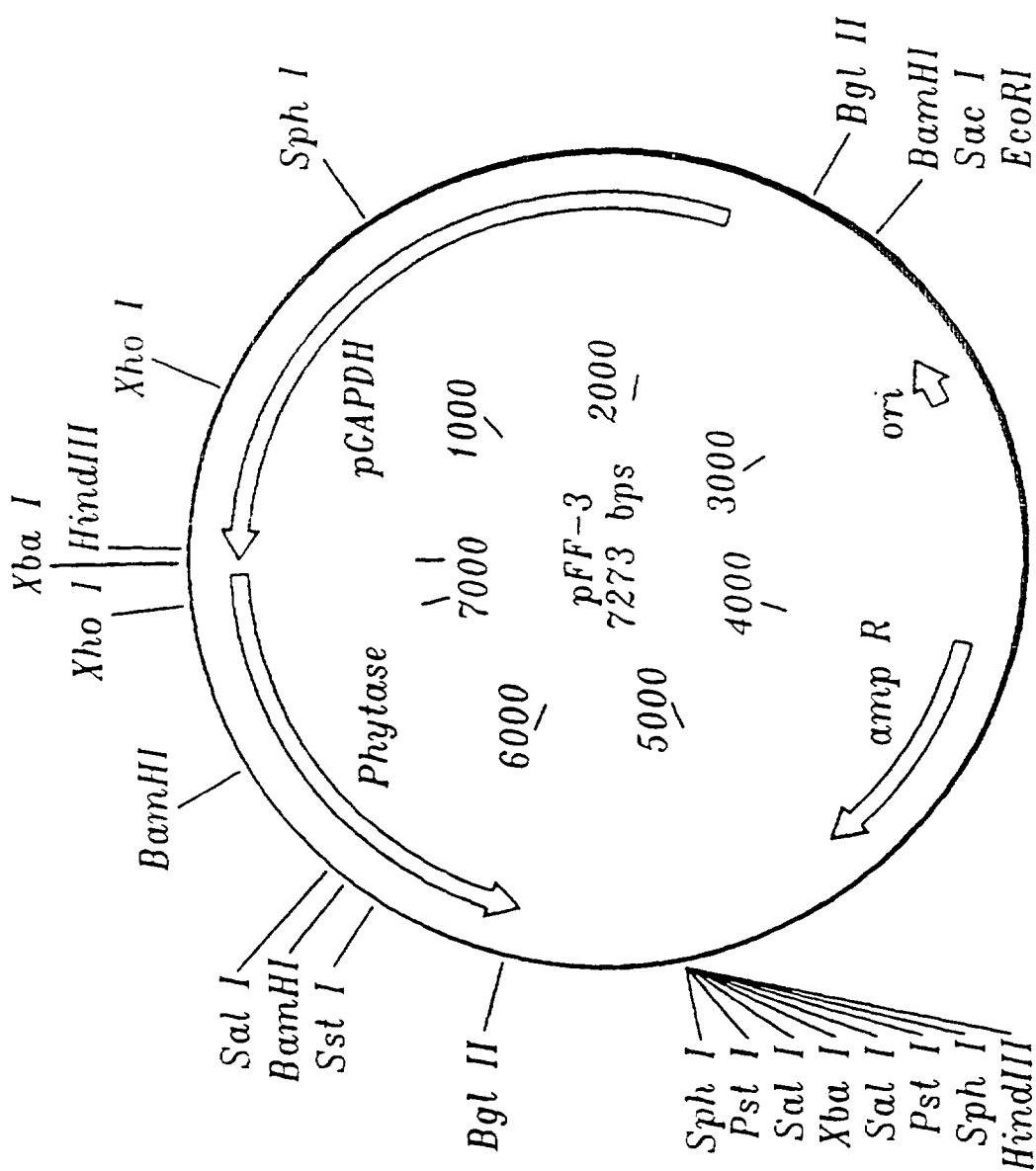


Fig. 10c.

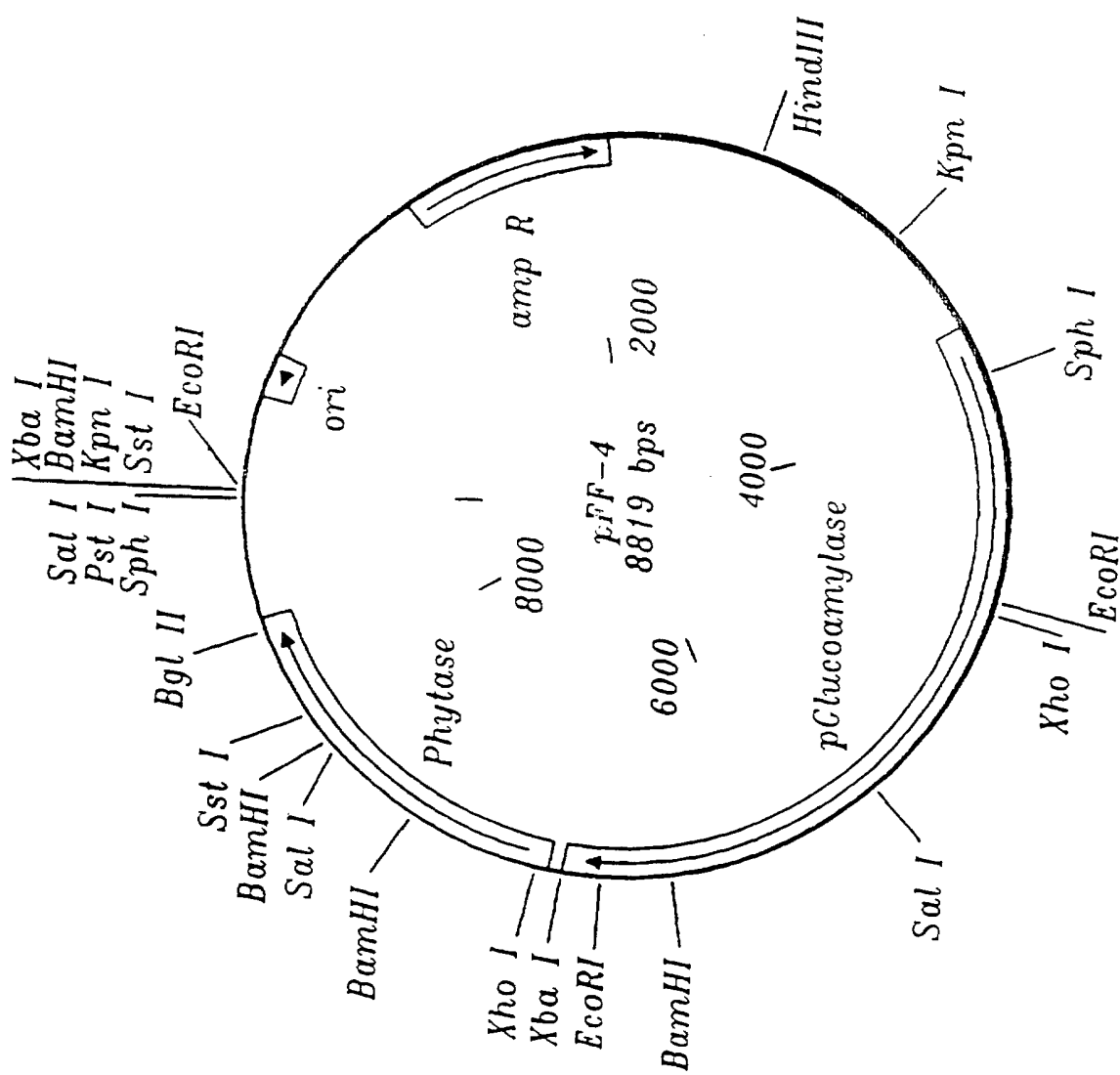


Fig. 100.

>sp1

1 GCATGCTGGA CCGCAATCTC CGATCGCGG GTATAAAGG TCCICCAAC CCCCTCGGT CGATATGTAC CCGCTCGTC ATCTCAATC CTCTCGAGAG
 101 CACCTTCTCC AGCTTTTGTC AATTGTACCT TCGCAATGCC TCGCACTCTY CTCTACCCC TGGCTGTGG TCTGGCCAGG GGGCATCCG CTTTCTCTA
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 201 CCGCGCTGCC ATTCCTCAGT CAACCCAGGA GAAGCAGTTC TCTCAGAGT TCGCGATGG CTACAGCATC CTCAAGCACT ACGGTGGTAA CGGACCTTAC
 G A A I P Q S T Q E K Q F S Q E F R D G Y S I L K H Y G G N G P Y>
 301 TCGAGCGTG TGTCTACGG TATCGCTGG GATCCCCCGA CCAGCTGGCA GGTGATCAG GTCAATCATG TCAAGCGTCA CGGAGAGCGG TACCCGTCCC
 S E R V S Y G I A R D P P T S C E V D Q V I M V K R H G E R Y P S>
 401 CTTCAGCGCG CAAGGACATC GAAGAGGCC TGGCCAAGGT CTACAGCATC AACACTACTG AATACAAGG CGACCTGGCC TTCTGAACG ACTGGACCTA
 P S A G K D I E E A L A K V Y S I N T Y E Y K G D L A F L N D W T Y>
 501 CTACGTCCCT AATGAGTGT ACTACAAGC CGAGACCACC AGCGGCCCTT AGCGGGTTC GCTGAGCGG TACAACCATG GCAACGATTA CAAGGCTGGC
 Y V P N E C Y Y N A E T T S G P Y A G L L D A Y N H G N D Y K A R>
 601 TACGGCCACC TCTGGAACGG TGAGACGGTC GTGCCCTTCT TTTCTAGTGG CTACGGAGCT GTCAATGAGA CCGCCCGCAA GTTCGGTGAG GGTTCCTTG
 Y G H L W N G E T V V P F F S S G Y G R V I E T A R K F G E G F F>
 701 GCTACAACTA CTCACCAAC GCTGCCCTCA ACATCATCTC CGAGTCCGAG GTCAATGGGG CGGACAGCTT CACGCCACC TGTGACACCG ACAACGACCA
 G Y N Y S T N A A L N I I S E S E V H G A D S L T P T C D T D N D Q>
 801 GACCACCTGC GACAACCTGA CTTACCAGT GCCCCAGTTC AAGGTGGCTG CTGCCGCCCT AAACCTCCAG AACCCCGGCA TGAACCTCAC CGCATCTGAT
 T T C D N L T Y Q L P Q F K V A A A R L N S Q N P G M N L T A S D>
 901 GTCTACAACC TGATGGGTAT GTGATTACGG TACAATCATT GGCTCAACC TCCAGCTGAC AGCATCTAG TTATGGCTC CTTGAGCTC AATGCTGCTC
 V Y N L M> V M A S F E L N A R>

Fig. 11A.

001 CCTCTCCAA CTGGATCAAC GCCTTTACCC AGGACGAATG GGTACGCTC GGTACGTTG AGGATTGAA CTACTACTAC TCGGCTGGGT GAGTTACCA
 P F S N W I N A F T Q D E W V S F G Y V E D L N Y Y Y C A G>

 1101 TTTGATCCAT TATTTGCTTG GATCAGCTAA CGATCGATAG TCCCGGTGAC AAGAACAATG CTGCTGTGG TCGCTCTAC GCCAACGCCA GTCTCACCCCT
 P G D K N M A A V G A V Y A N A S L T L>

 1201 CTTGAACCAG GGACCAAGG AAGCGGCTC CTGTTCTTC AACTGTACG TCTCGGCAG AATCAGAGTC TCACAAAAAG AAATCTTCA CTAACATATA
 L N Q G P K E A G S L F F N F>

 1301 GTAGTGCCCA CGACACCAAC ATCACCCCA TCTCGCGC CCTAGGCTC CTCATCCCA AGGAGGACCT TCCTTTGAC CGGGTGGCT TCGGAACCC
 A H D T N I T P I L A A L G V L I P N E D L P L D R V A F G N P>

 1401 CTACTCGATC GGCAACATCG TGGCCATCTG ACCATCGAGC GTCTCAGCTG CCAGGCCACC GCCCTCTCGG ACGAGGTAC CTACGTGGT
 Y S I G N I V P M G G H L T J E R L S C Q A T A L S D E G T Y V R>

 1501 CTGGTGCTGA ACGAGGTGT ACTCCCTTC AACGACTGCA CTTCCGGACC GGGCTACTCC TGGCTCTGG CCACTACAC CTCCATCTG AACAGAATC
 L V L N E A V L P F N D C T S G P G Y S C P L A N Y T S I L N K N>

 1601 TGGCAGACTA CACGACCACC TGAATGTCT CTGGTCTTA CCCGAGTAT CTGAGCTTCT GGTGGAACCTA CAACACCACG ACGAGCTGA ACTACCGCTC
 L P D Y T T T C N V S A S Y P Q Y L S F W W N Y N T T T E L N Y R S>

 1701 TAGCCCTATT GCCTGGCAGG AGGGTGATG TATGGACTAG ATGCAGAGG GTAGGTCCG GGATACTTA GTGATGATTG ATATTCAAGT TTGGTGGTGA
 S P I A C Q E G D A M D *>

 1801 CGATCACCTY GTTAATAGTC TTGTACAGTC ATACGGTGAA TGTAAATAT GATAATAGCA ATGATACATG TTGGAATCTC GTTTGTCT TGTGTGCT

 1901 AGGGCTTTG GGGGTGTATT TTAGGCGT AGACTTATT TCAATTCGTG TATAATGCGG TCAGTAAATG AATCATCAAT TATTCAATG CAATGCTGA

 2001 TACGTGAAC TATTTGGTTA AGCGCAGCT ACTAGCTGAC TGGTTGGTTA CTTCTGTGT ACACCGCATG C

>Sph1

Fig. 11B.

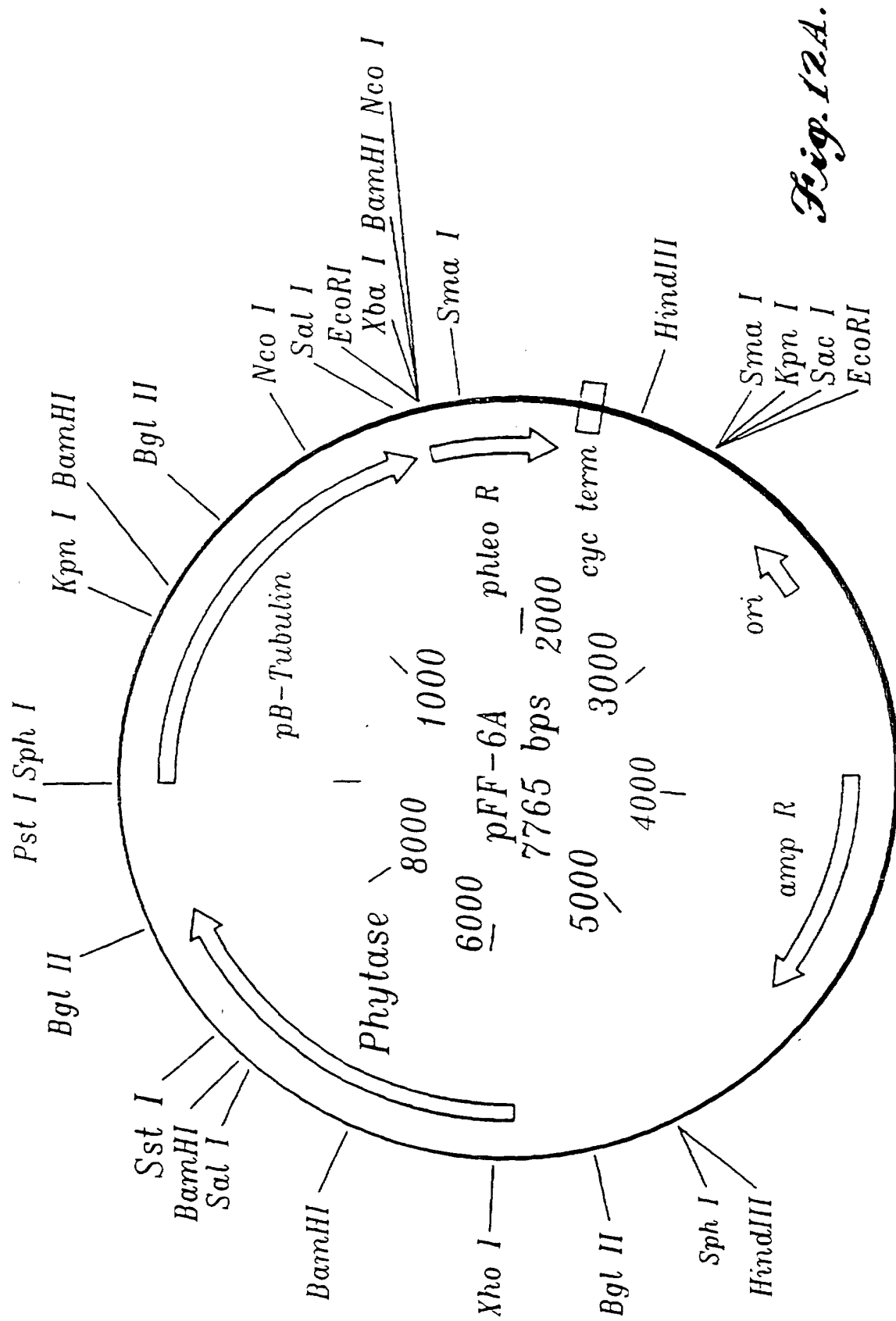


Fig. 12A.

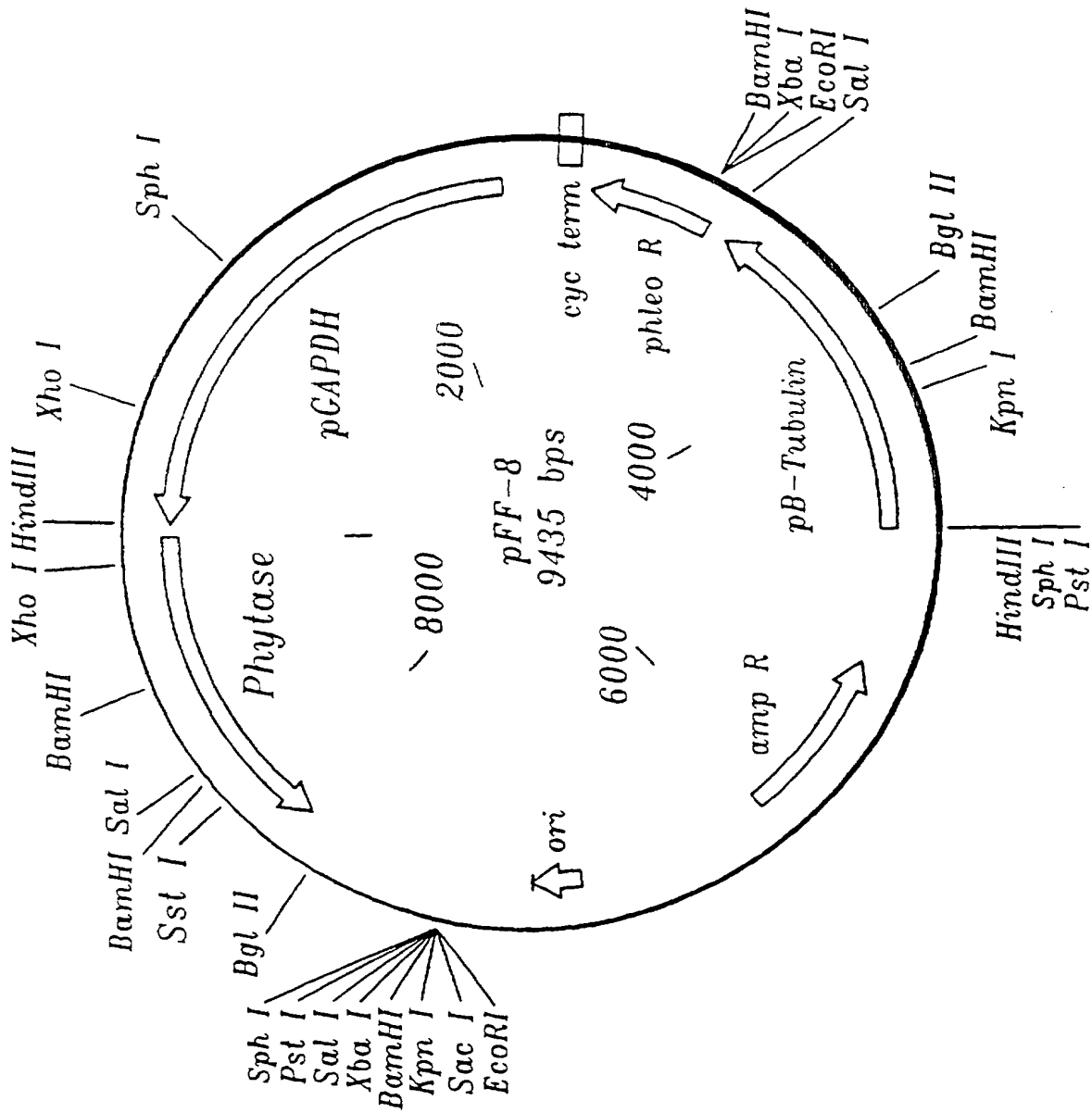
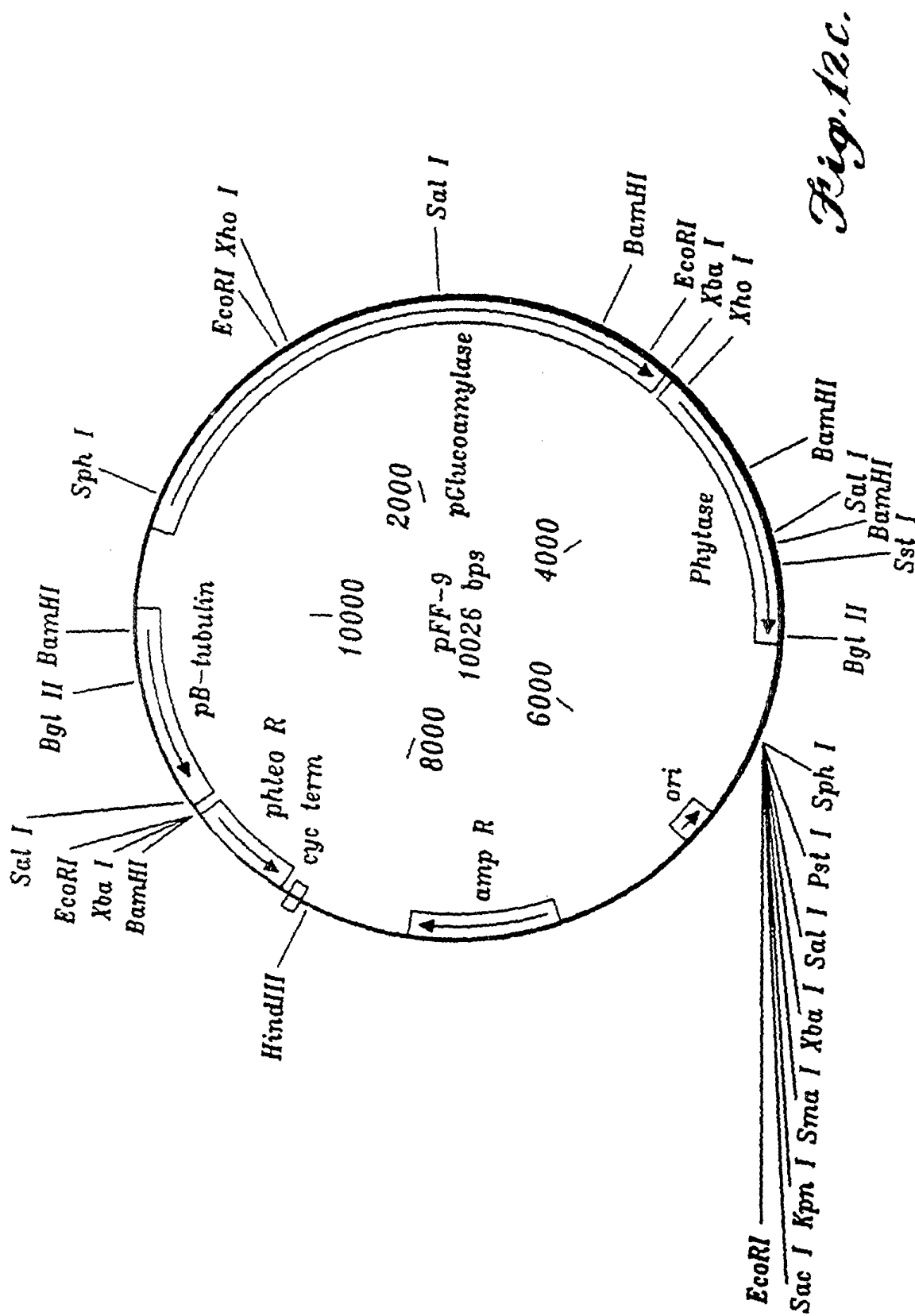


Fig. 12B.



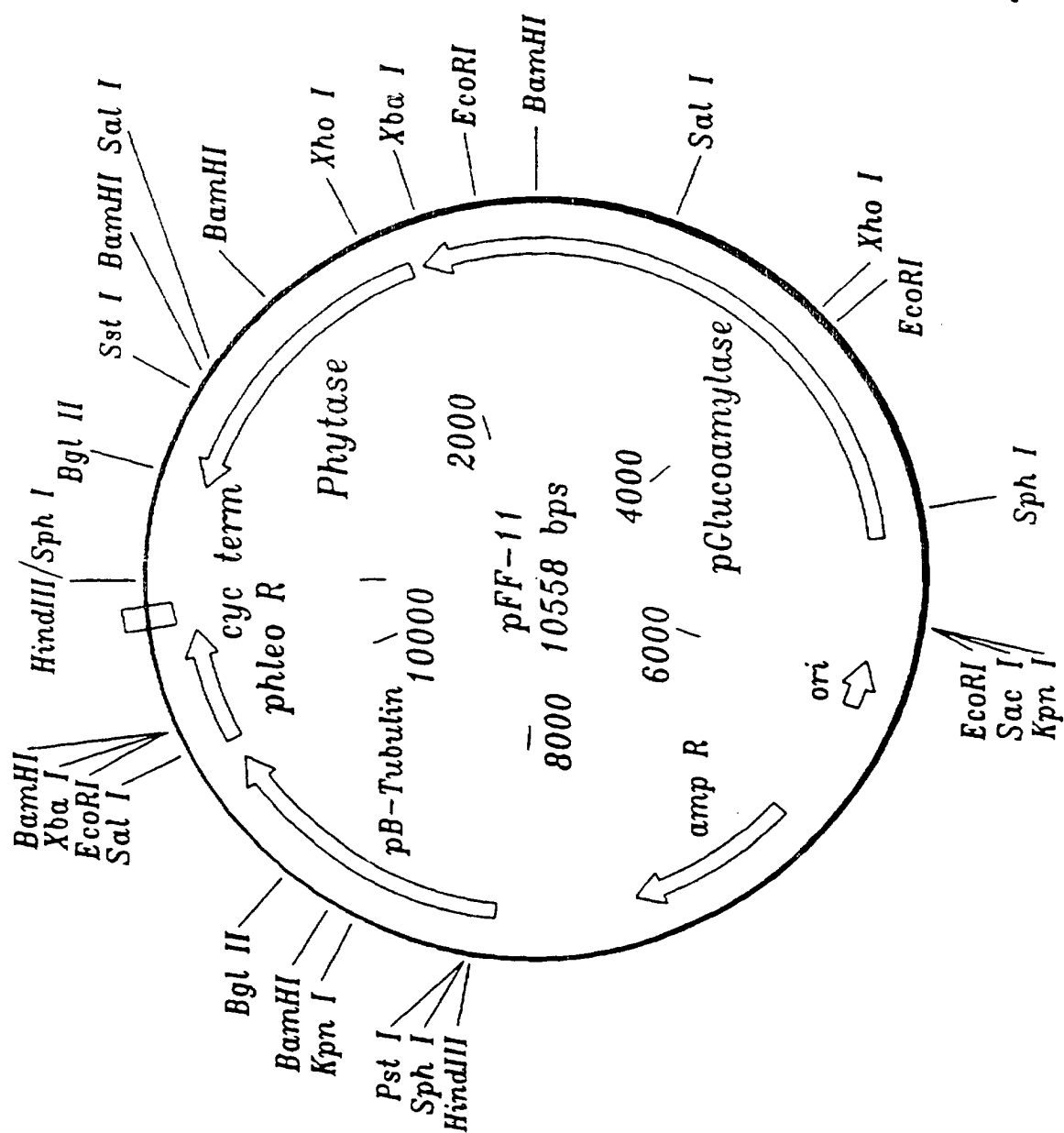


Fig. 12d.

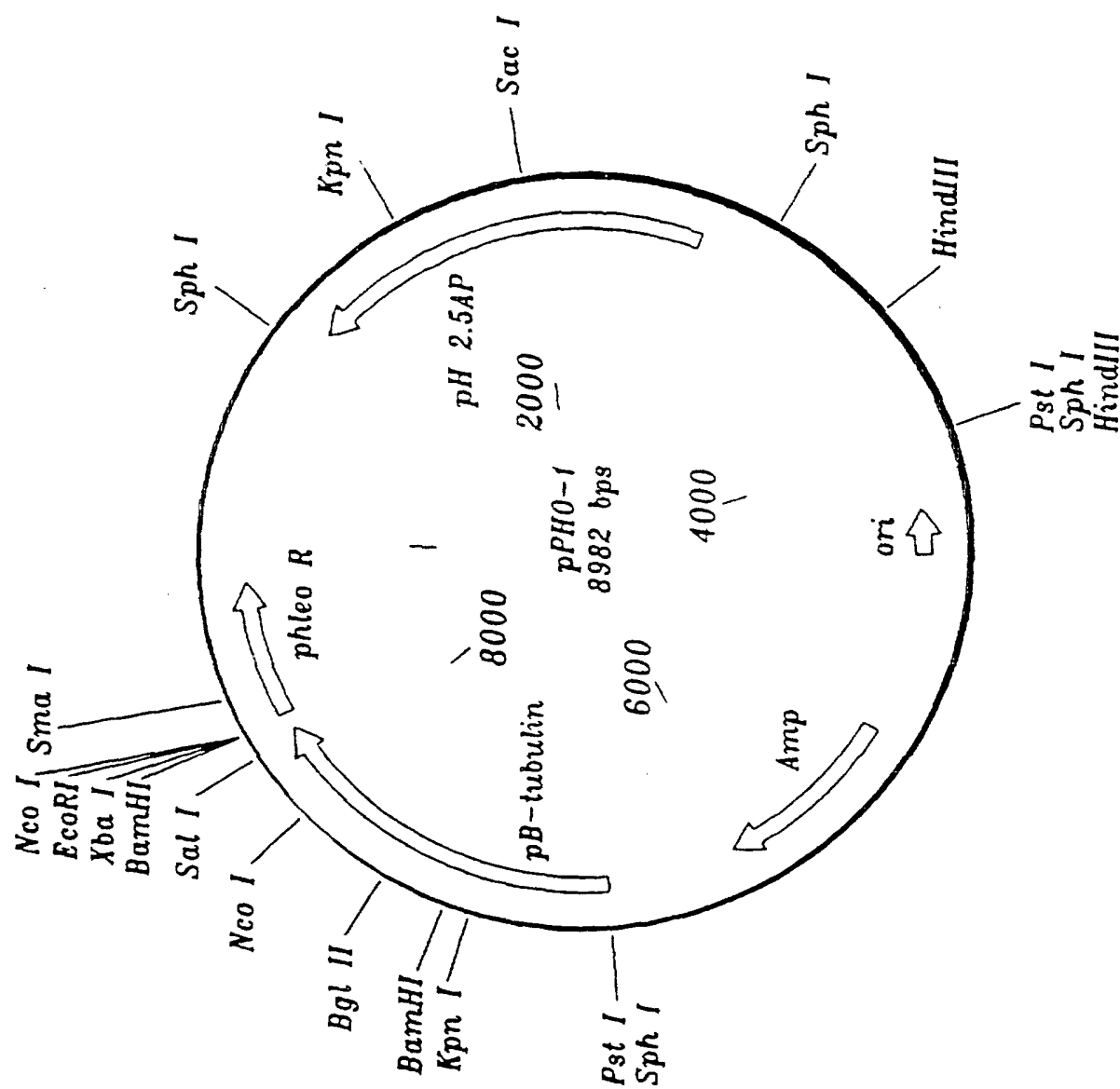


Fig. 13A.

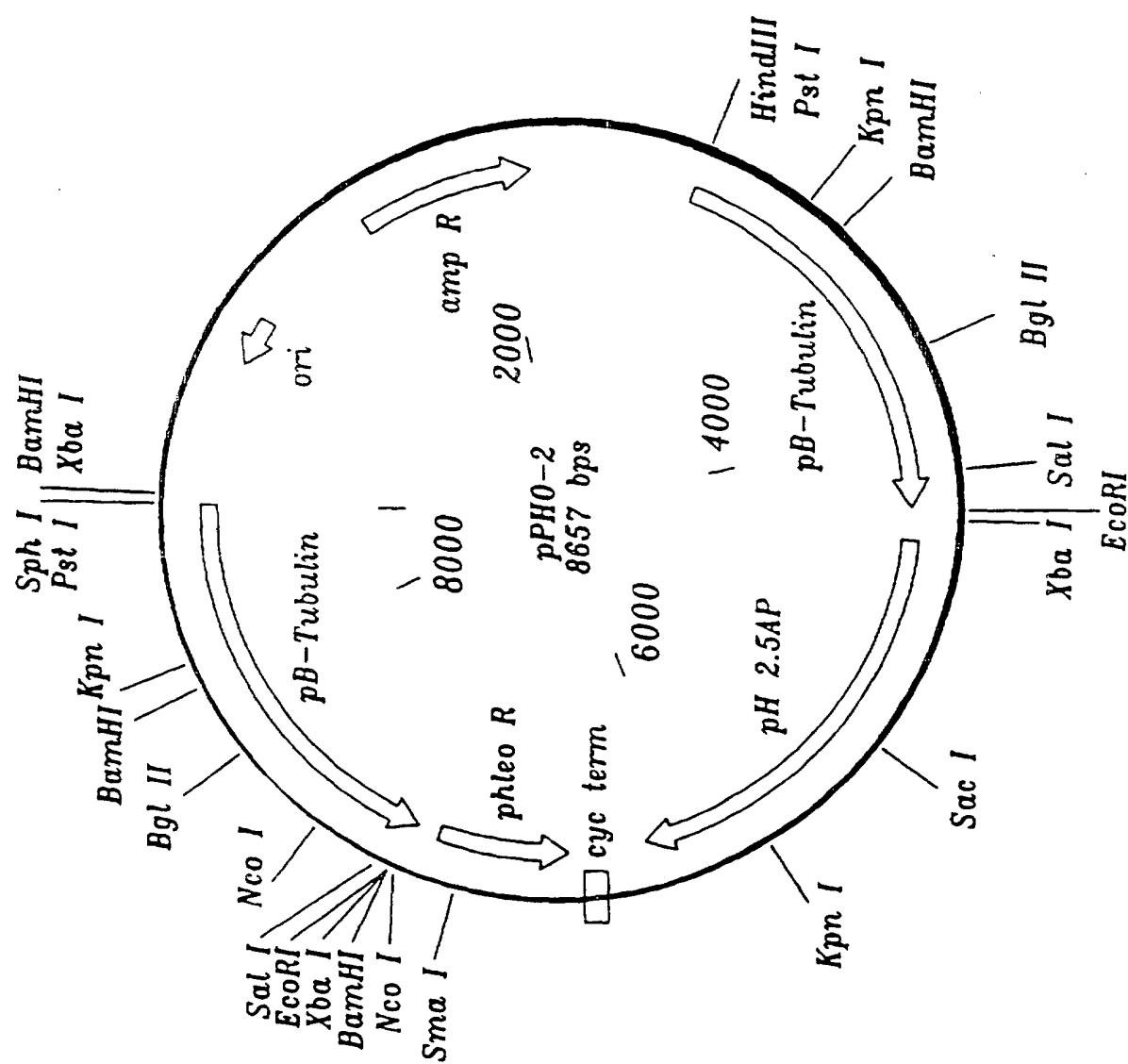


Fig. 13B.

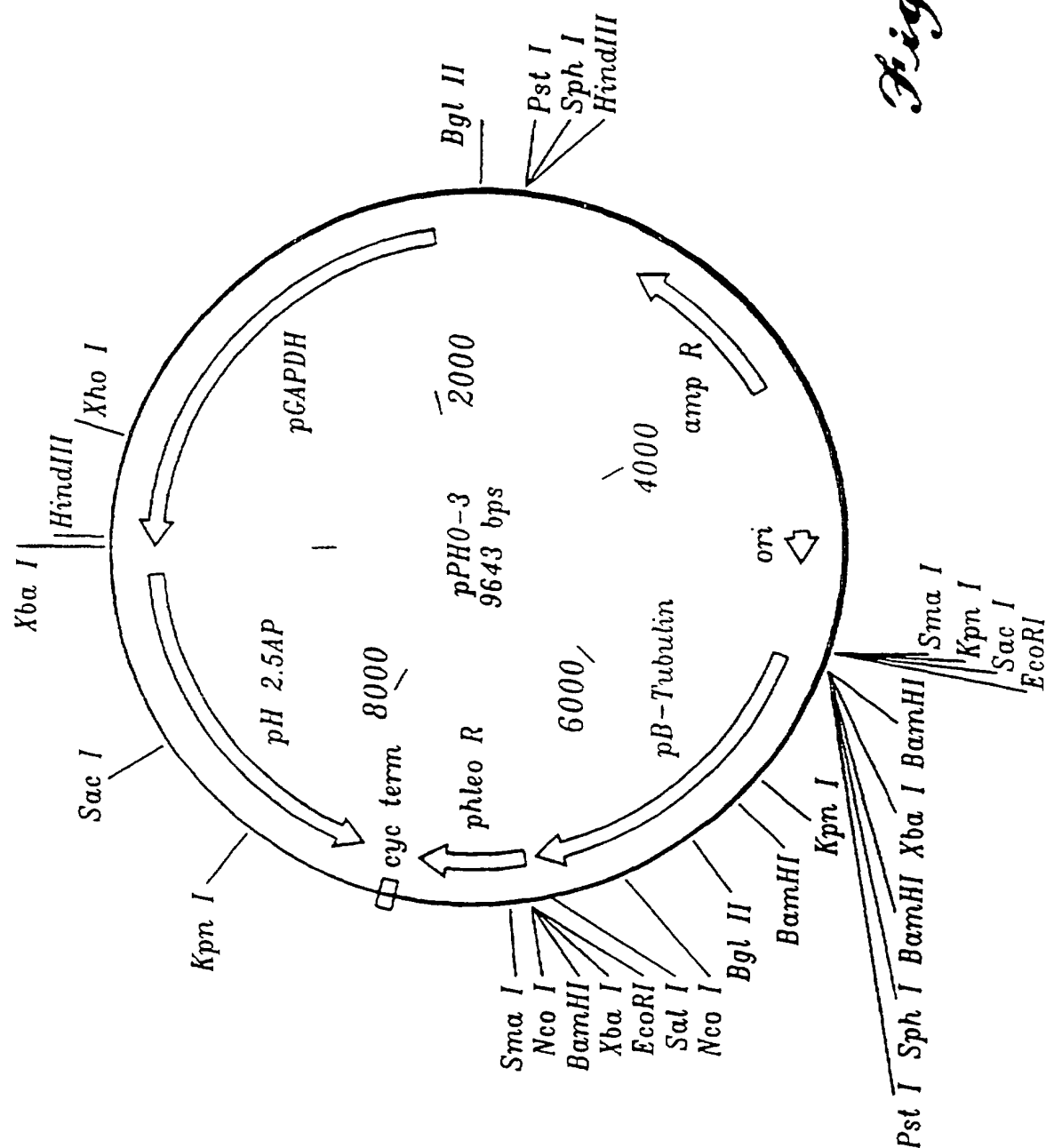


Fig. 13C.

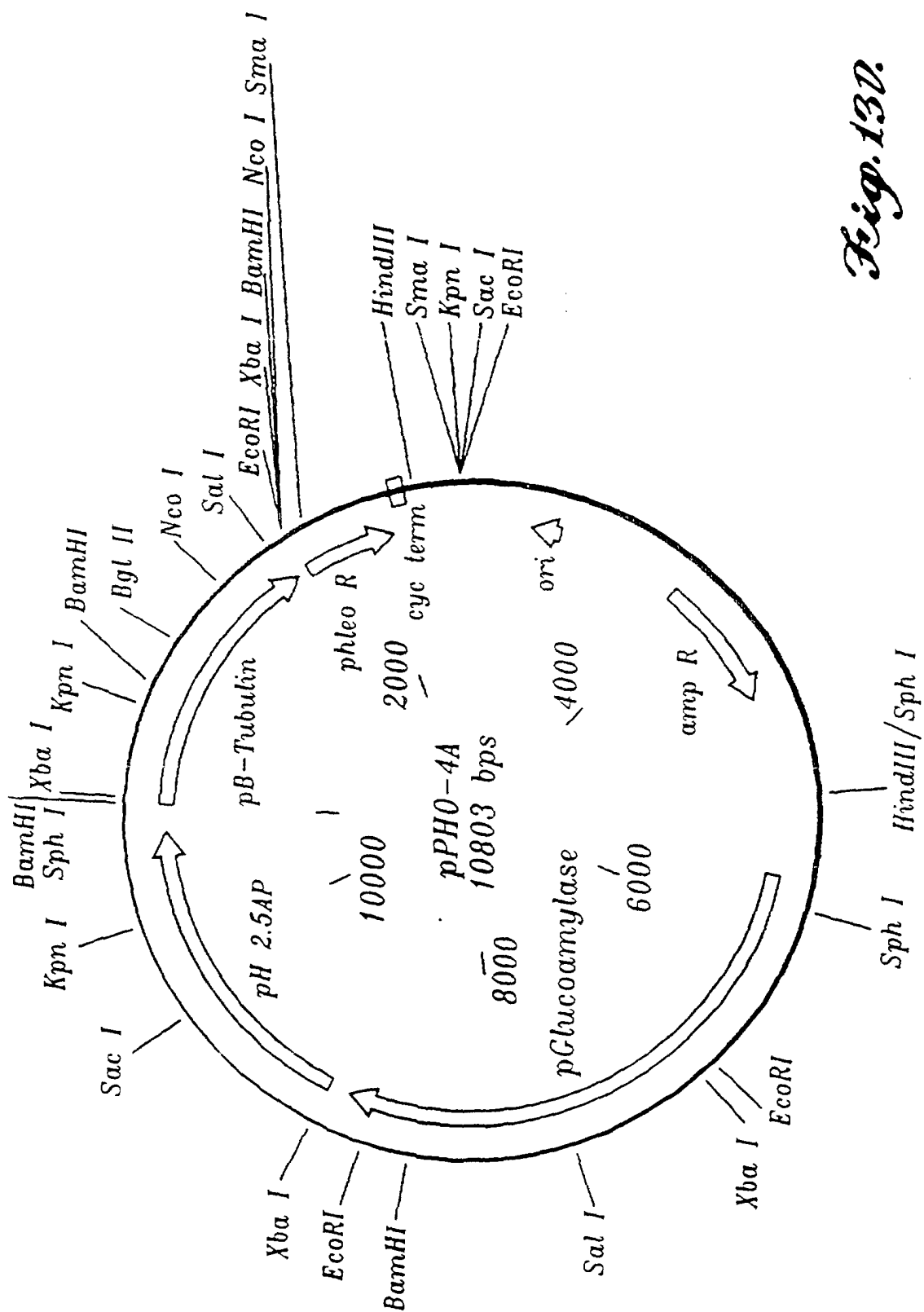
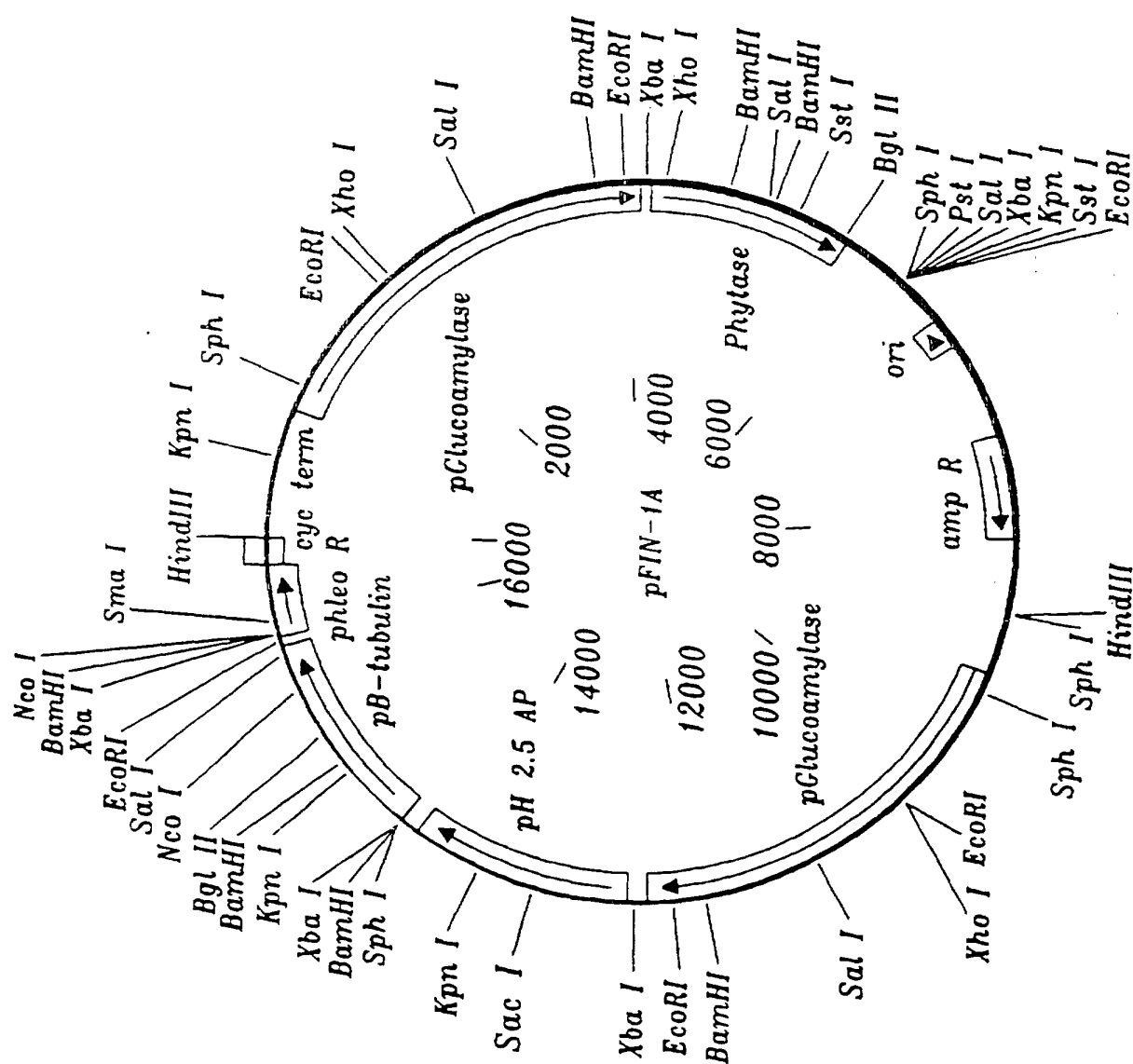


Fig. 13D.

Fig. 14A.



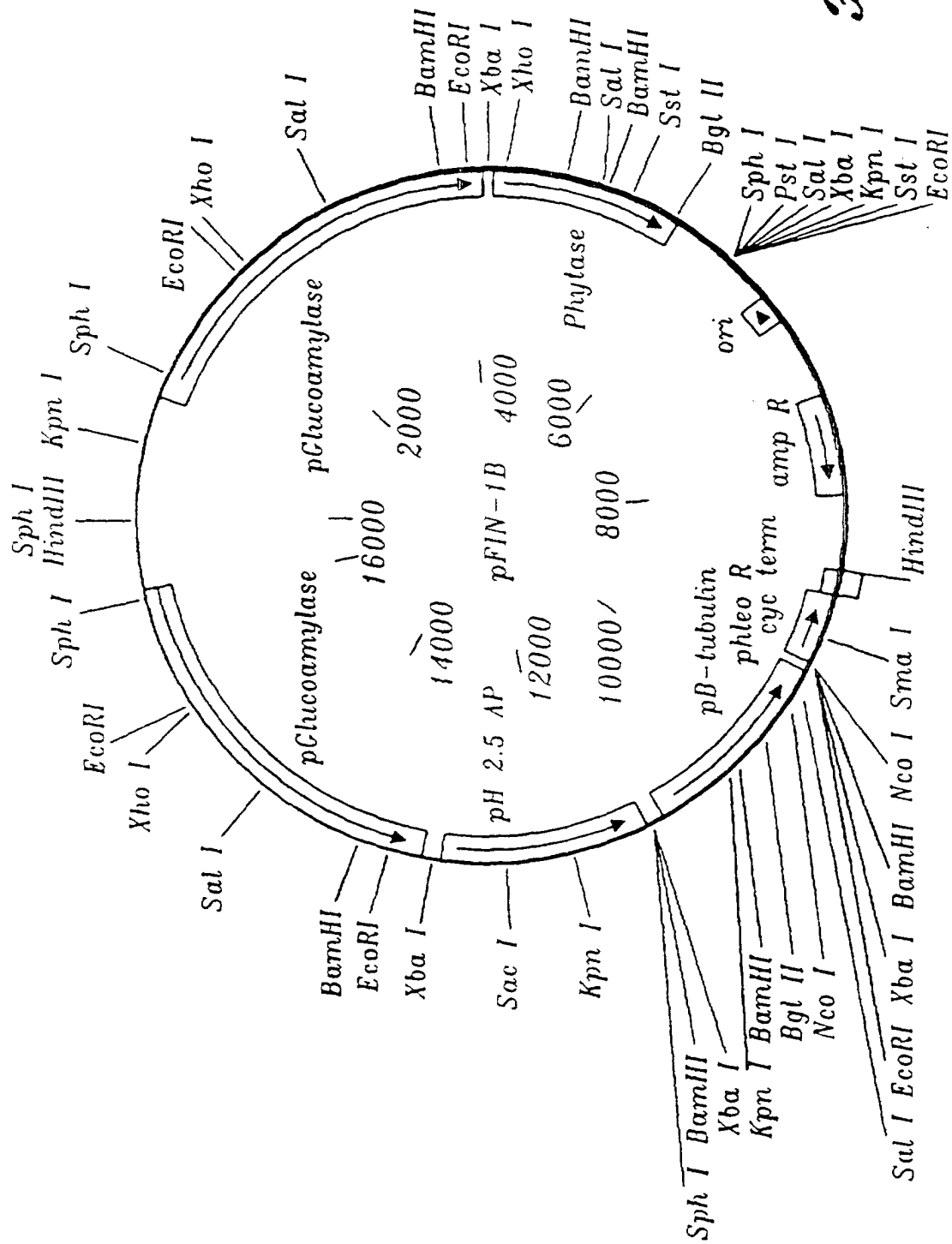


Fig. 14B.

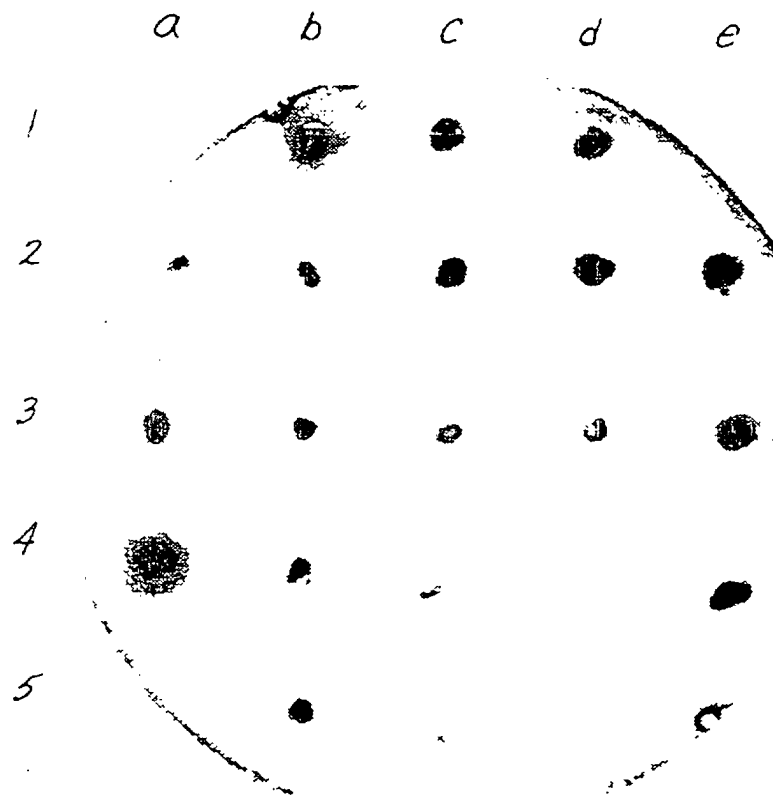


Fig.15.

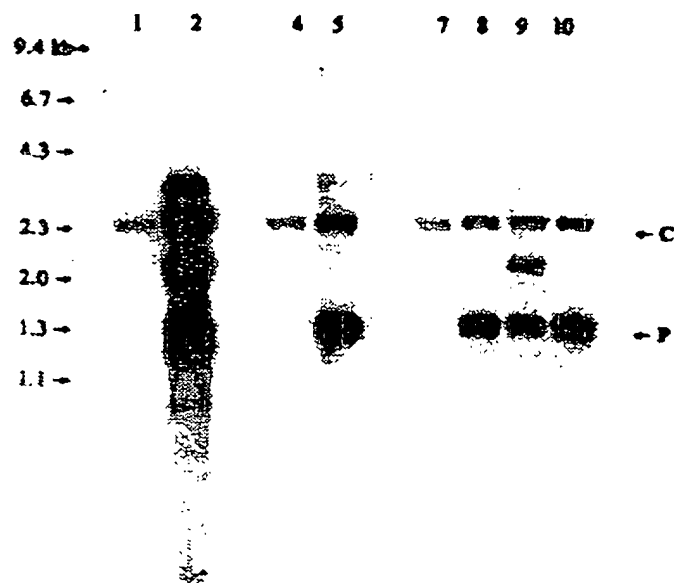


Fig.16A.

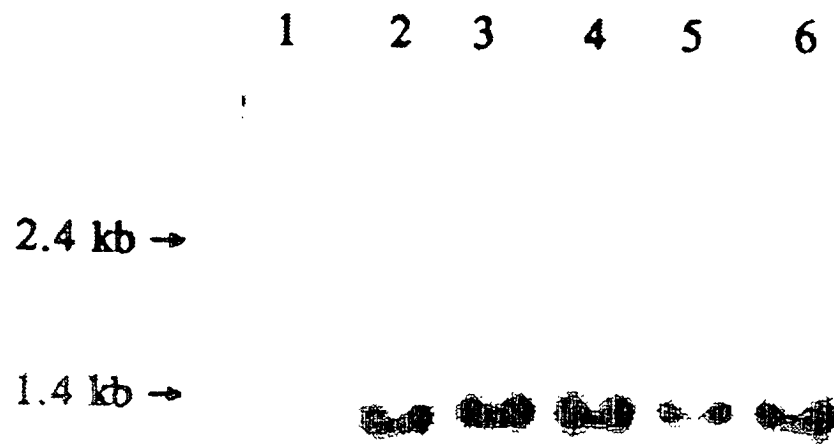


Fig. 16 B.

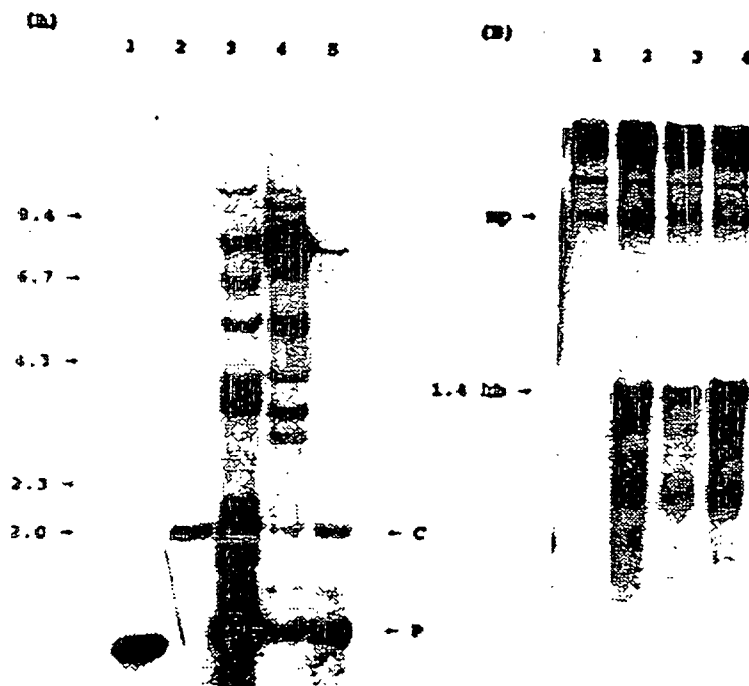


Fig. 17A.

Fig. 17B.

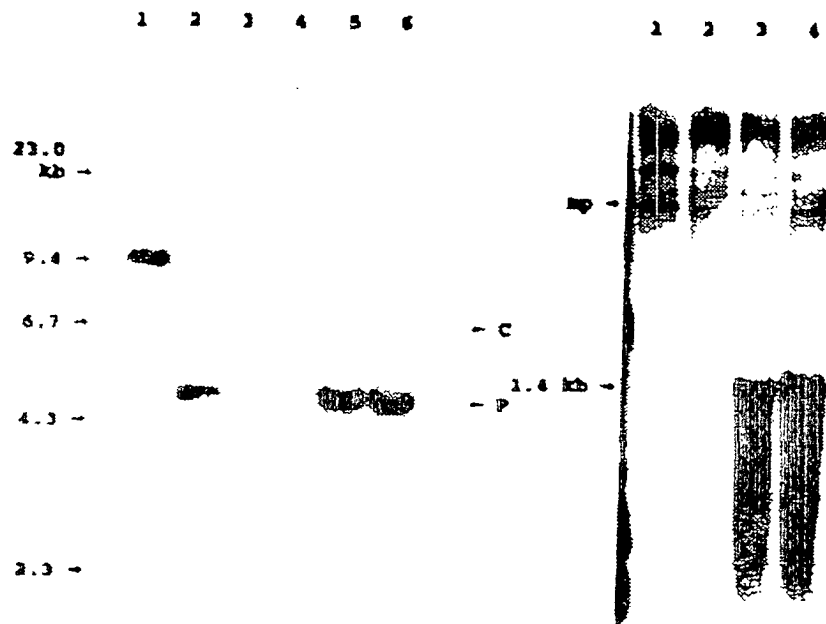


Fig. 18A.

Fig. 18B.

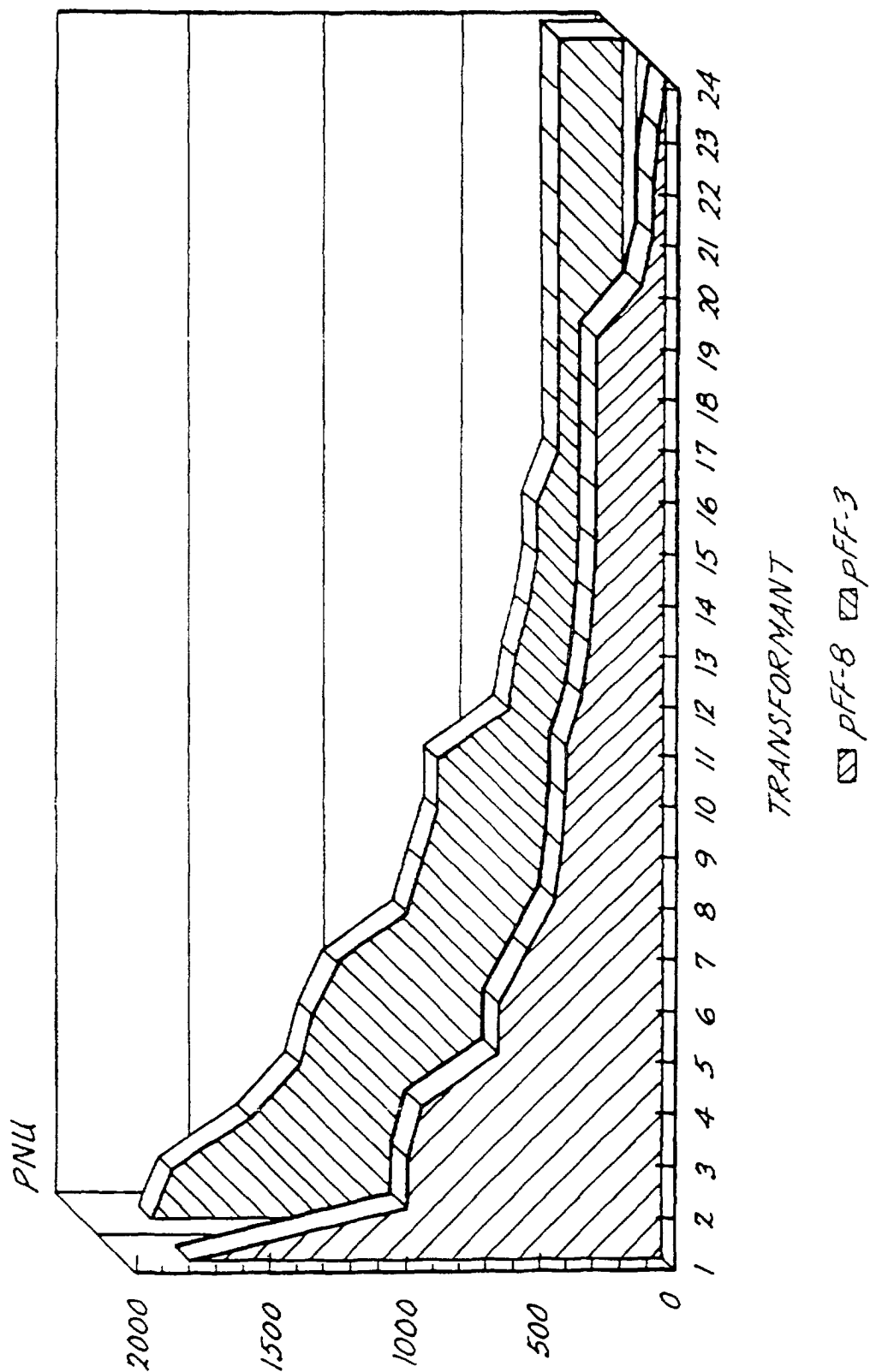


Fig. 19.



Fig. 20.